BRITISH JOURNAL OF CANCER

VOL. XII MARCH, 1958 NO. 1

BRONCHIAL NEOPLASMS AMONG THE CEYLONESE

G. H. COORAY AND N. D. G. LESLIE

From the Department of Pathology, University of Ceylon

Received for publication December 12, 1957

REPORTS on studies of lung cancer have originated chiefly in western countries where heavy industry has reached a high stage of development and where the smoking of tobacco is very freely indulged in. It was therefore thought that a study of the features of bronchial carcinoma in Ceylon, a non-industrial country where the customs and habits of people differ from those of the west, might be helpful in the understanding of the general problem of carcinoma at this site.

The data for this study were obtained from the records of cases admitted to the Thoracic Unit of the General Hospital, Colombo, to which all cases of suspected pulmonary neoplasms are referred from various parts of Ceylon.

Incidence.—According to biopsy records there were 22 cases of bronchial carcinoma and 5 cases of bronchial adenoma during the five year period 1952–56.

In a series of 2562 consecutive autopsies performed in Colombo during this same period there were only 5 bronchial carcinomas—an incidence of 0·2 per cent.

Of a series of 1149 admissions to the Thoracic Unit during 1954–56, 22 were carcinomas of the bronchus, i.e. 1·9 per cent of the admissions.

Sex and age incidence.—Sixteen of the bronchial carcinomas occurred in men and 6 in women—a proportion of 2·7 : 1. Three of the bronchial adenomas occurred in men and 2 in women.

In the 16 cases of carcinomas where the age had been recorded, 2 occurred in each of the third and fourth decades, 5 in each of the fifth and sixth decades and 2 in the seventh decade. All the bronchial adenomas occurred in adults above the age of 30 years.

Histological types

(1) *Bronchial carcinoma.*—According to the prevalent structure, we have classified the neoplasms into three main types: (*a*) epidermoid carcinoma, (*b*) anaplastic carcinoma, (*c*) adenocarcinoma.

(*a*) Epidermoid carcinoma was the most prevalent type. Nine out of the 22 cases (41 per cent) belonged to this type (Fig. 1, 2, 3).

(*b*) Anaplastic carcinoma comprised 8 out of the 22 cases (36 per cent) (Fig. 4).

(*c*) Adenocarcinoma the rarest type, was seen in only 3 cases (14 per cent.).

(*d*) Unclassified: 2 cases where malignant cells were found in the pleural fluid (9 per cent).

These various histological types were not peculiar to any age or sex.

(2) *Bronchial adenoma.* Of the 5 cases, 4 belonged to the carcinoid type of bronchiolar adenoma and one was an alveolar cell adenoma (Fig. 5, 6).

DISCUSSION

(a) *Bronchial carcinoma*

Incidence.—The average number of cases of bronchial carcinoma diagnosed by bronchial biopsy in Ceylon appears to be 4·4 per year, which is much less than the number diagnosed by this method in central laboratories in the United Kingdom, Europe and America.

Hospital records too suggest a much lower incidence of bronchial carcinoma in Ceylon as compared with western countries. Maxwell and Nicholson (1930) recorded that in the years 1924–28, there were 318 cancers in their series of 1851 cases—an incidence of 14·15 per cent. In Ceylon, out of 1149 admissions, there were only 22 carcinomas—an incidence of 1·9 per cent.

Necropsy figures for bronchial carcinoma given in Table I show a much lower incidence in Ceylon and some of the tropical countries, e.g. India, Indonesia and East Africa. Moreover, while the incidence of bronchial carcinoma appears to be increasing annually in western countries (Doll, 1953; Simpson, 1929; Clemmesen 1953), in Ceylon the number of such cases seen each year appears to be rather constant.

TABLE I

Authors	Place	Year	Total necropsies	Total cancers	Percentage of cancers
Duguid (1927)	Manchester	1916–20	910	22	2·42
Simpson (1929)	London	1925	585	12	2·05
Dick (1940)	Glasgow	1934–38	2216	87	3·98
Ochsner and De Bakey (1941)	New Orleans	1938	825	17	2·06
Bryson and Spencer (1951)	London	1945–47	—	—	27·2
Nath and Grewal (1935)	India	—	—	—	0·1
Kouwenaar (1950)	Indonesia	—	—	—	0·1
Present series	—	1952–56	2562	5	0·2

Histology.—The same histological types met with in other countries are seen in Ceylon. In other words, when the bronchial mucosa is stimulated by a carcinogenic agent the response is much the same, the only difference being that the stimulus does not act with the same degree of frequency as in European countries. The low incidence of bronchial carcinoma as testified by clinical, post mortem and biopsy records indicate that the lung is not exposed to the same degree of irritation by carcinogens.

Age and sex incidence.—In Ceylon, as in other European countries, lung cancer appears to be a disease of late life—10 of our cases occurring in the fifth and sixth decades. The proportion of men to women affected in western countries as given by different workers, varies from 3·7 : 1 to 6·3 : 1. Our sex incidence of 2·7 : 1 suggests that although males are more frequently affected, there is not that degree of preponderance of males over females as exists in European peoples.

(b) *Bronchial adenoma*

As in other countries, (Willis, 1953) bronchial adenoma affects the sexes in this country almost equally, but this neoplasm is seen more frequently than in Europeans. Willis (*loc cit.*) saw only 6 bronchial adenomas during a period in which 84 necropsies on bronchial cancer were performed—an incidence of 7 per cent, whereas in this series we have met with 5 such tumours during a period in which only 22 carcinomas were examined—an incidence of 21 per cent. No explanation can be offered for the increased frequency with which this tumour is met with in the Ceylonese.

Tobacco and industry

Table II shows the tobacco consumption according to the import of all tobacco products to Ceylon, as well as the cigarette consumption among the population for the period 1952–56.

TABLE II

(Population of Ceylon according to 1955 Census, 8,098,637.)

	1952	1953	1954	1955	1956	England and Wales Doll (1953) 1900–50
All tobacco imports (lb.) from Ceylon Customs returns	3,873,017	3,874,699	3,722,651	3,951,135	4,085,840	—
lb. consumed per person	0·48	0·48	0·46	0·49	0·50	1·85–4·5
Tobacco consumed in cigarettes (lb.)	3,780,000	3,750,000	3,300,000	3,360,000	3,570,000	—
lb. consumed per person in cigarettes	0·47	0·46	0·41	0·42	0·44	0·15–4·0

A study of these figures reveals that both tobacco and cigarette consumption, in Ceylon are far below that in England and Wales. Moreover, while in England and Wales, tobacco and cigarette consumption are showing a tendency to increase annually (Doll, *loc. cit.*) the consumption in Ceylon appears to have remained more or less stationary during the period 1952–56.

In addition, in Ceylon, there are no heavy industries. Hence the comparatively low incidence of bronchial carcinoma in Ceylon supports the generally prevalent concept that inhaled carcinogens from such sources as tobacco and from an atmosphere polluted by smoke from industrial sources, play a major role in the causation of this neoplasm.

SUMMARY

1. In a period of 5 years (1952–56) only 22 cases of bronchial carcinoma (average 4·4 per year) were diagnosed by bronchial biopsy at the Thoracic Unit of the General Hospital, Colombo, to which all suspected cases are referred to from different parts of the island. During this same period there were 5 cases of bronchial adenoma.

The incidence of bronchial carcinoma in autopsy material during this same period was 0·2 per cent of total necropsies.

From clinical records it was observed that 1·9 per cent of the total admissions to the thoracic unit were bronchial carcinomas.

No explanation can be offered for the fairly high incidence of bronchial adenoma.

2. Bronchial carcinoma appears to be a disease of later life—10 out of the 22 cases occurring in the fifth and sixth decade.

3. The sex incidence, 2·7 males to 1 female, does not show such a marked disproportion between males and females as is shown in western countries.

4. Most of the histological types met with in European countries were seen here.

5. The low incidence of bronchial carcinoma is attributed to the relative freedom from atmospheric pollution by substances emanated from industrial sources and to less cigarette smoking among the population.

We wish to thank Mr. A. T. S. Paul, Thoracic Surgeon, General Hospital, Colombo, for much assistance and Mr. P. D. S. Amarasekara for the photographs.

REFERENCES

Bryson, C. C. and Spencer, H.—(1951) *Quart. J. Med.*, **20**, 173.

Clemmesen, J.—(1950) In symposium of geographical pathology and demography of cancer (WHO and UNESCO) cited by Doll, R., 1953, *Brit. med. J.*, ii, 585.

Dick, J. C.—(1940) *Glasgow med. J.*, **16**, 63.

Doll, R.—(1953) *Brit. med. J.*, ii, 521.

Duguid, J. B.—(1927) *Lancet*, ii, 111.

Kouwenaar, W.—(1950) In symposium of geographical pathology and demography of cancer (WHO and UNESCO) cited by Doll, R., 1953, *Brit. med. J.*, 585.

Maxwell, J. and Nicholson, W. A.—(1930) *Quart. J. Med.*, **24**, 29.

Nath, V. and Grewal, K. S.—(1935) *Indian J. med. Res.*, **23**, 149, cited by Doll, R., 1953, *Brit. med. J.*, ii, 585.

Ochsner, A. and de Bakey, M.—(1941) *Arch. Surg.*, **42**, 209.

Simpson, S. L.—(1929) *Quart. J. Med.*, **22**, 413.

Willis, R. A.—(1953) ' Pathology of Tumours '. London (Butterworth and Co.).

EXPLANATION OF PLATES.

Fig. 1.—Gross specimen showing occlusion of bronchus by carcinoma.

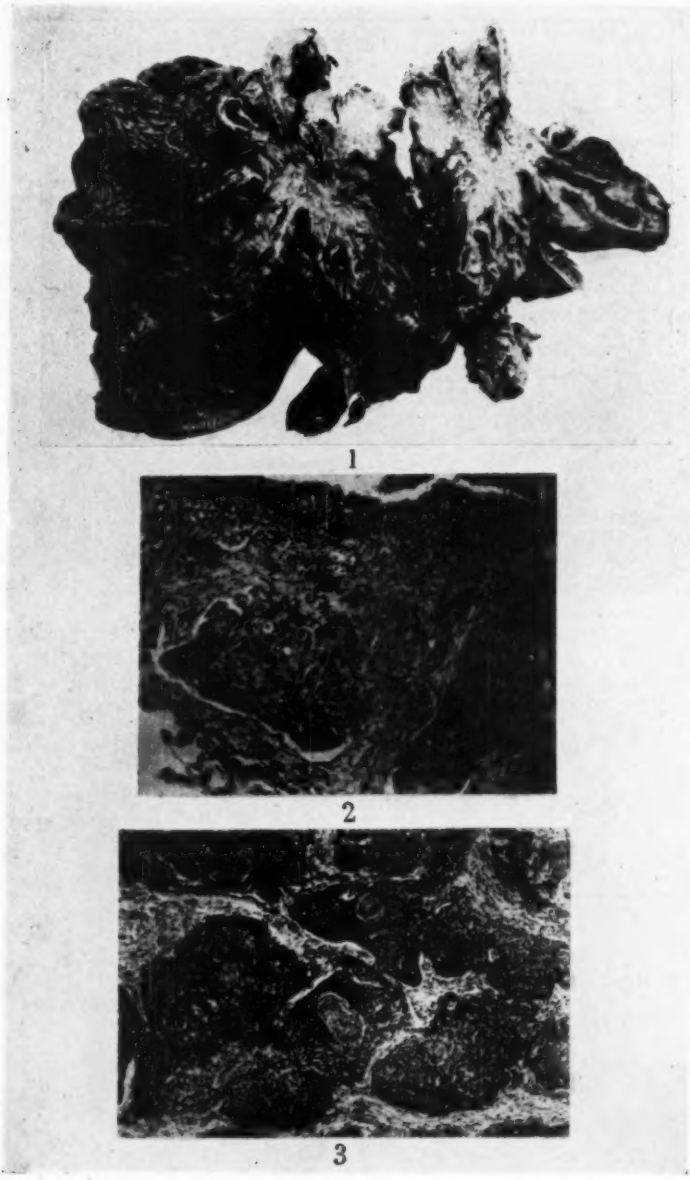
Fig. 2.—Section from growth in Fig. 1 showing squamous cell type of structure. H. & E. × 100.

Fig. 3.—Epidermoid carcinoma showing " pearl " formation. H. & E. × 50.

Fig. 4.—Anaplastic carcinoma " oat cell " type. H. & E. × 185.

Fig. 5.—Gross specimen showing growth (bronchial adenoma) in lingula.

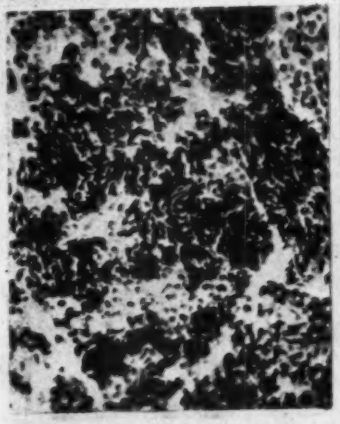
Fig. 6.—Alveolar structure of growth shown in Fig. 5. H. & E. × 55.



1

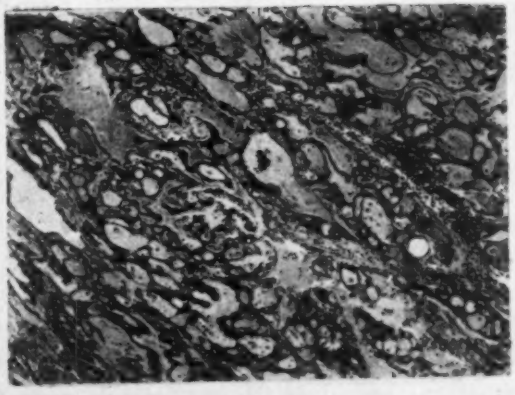
2

3



4

5



6

THE SPECIES NON-SPECIFICITY OF GLOBULINS IN THE GLOBULIN-FLUORESCEIN STAINING OF TISSUES

E. S. J. KING, P. E. HUGHES AND C. J. LOUIS

From the Department of Pathology, University of Melbourne, Melbourne, Australia

Received for publication November 27, 1957

MANY observers have suggested that the malignant cell differs from its normal counterpart in the loss or modification of a growth-controlling component but it was not until the work of Miller and Miller (1947) that firm experimental support for such an hypothesis was advanced. This work indicated that, at least as far as rat liver is concerned, the cellular constituent involved is protein in nature.

The results of Weiler's observations (1952, 1956) provided independent serological and histological support for the concept of the loss of a cellular constituent during aminoazo dye carcinogenesis in the rat liver. Weiler injected particulate antigens prepared from rat liver into rabbits and obtained antisera which, after repeated absorption with rat kidney particulates, still reacted in complement fixation test with the homologous rat liver but failed to react with rat kidney particulates. He found that a serum prepared in this way, which he regarded as a liver "organ-specific antiserum", did not react with particulate diagnostic "antigens" prepared from rat hepatomata and concluded that the tumour cells had lost their "organ-specific antigen". Further support of these results was obtained by application of the fluorescence antibody technique of Coons and Kaplan (1950). Weiler found that fluorescein-globulin conjugates prepared from "organ-specific anti-rat liver" rabbit globulin stained normal rat liver sections but did not stain sections of rat hepatoma.

Hughes, Louis, Dineen and Spector (1957), using Weiler's methods, were unable to obtain an organ-specific antiserum. At the same time the differential staining of normal rat liver and rat hepatoma sections by the use of a fluorescein-conjugated rabbit globulin fraction was completely confirmed (Hughes, 1957). At first serum obtained from rabbits which had previously been injected with homogenates of rat tissues was used. However, it was found that serum from rabbits which had not been so injected was equally effective as a stain. These control sera did not fix complement in the presence of rat liver antigens.

The method was applied to naturally-occurring tumours both in man and in animals. At first the sera from rabbits which had been injected with the appropriate tissues were used, but these were soon replaced by sera from uninjected animals. The technique was applied to various tumours and particularly to a series of carcinoma of the colon (Louis, 1957*b*) and it was also applied to a large series of cases of leukaemia (Louis, 1957*c*).

In view of these results it was clear that the staining could not be attributed to an antibody-antigen reaction and an alternative explanation was sought. In mixtures of proteins, anion-cation salt-like complexes are readily formed within the pH range between the isoelectric points of the components. The formation of these complexes between "stain globulin" and tissue proteins could account for these staining phenomena.

Since the γ-globulins as a whole represent a relatively homogeneous class of proteins the staining reactions of dye-globulin conjugates of various animals have been studied to determine if this staining property is a peculiarity of rabbit globulin or if it is a common property of globulin conjugates prepared from different genera, orders or even classes.

The results of an investigation using globulin conjugates of diverse origin are presented here. Most globulin fractions used were γ-globulin fractions prepared by cold ethanolic fractionation ; some other crude globulin fractions were prepared by ammonium sulphate fractionation. Globulin conjugates were prepared with both fluorescein and rhodamine.

MATERIALS AND METHODS

(1) *Preparation of globulin fractions*

Fowl, mouse and some rabbit globulin fractions were prepared by precipitation with 50 per cent saturated ammonium sulphate at 1° C. and subsequent dialysis against phosphate buffered saline pH 7·2.

Human γ-globulin, prepared by method 6 of Cohn, Strong, Hughes, Mulford, Ashworth, Melin and Taylor (1946), was obtained from the Commonwealth Serum Laboratories, Melbourne. Γ-globulin fractions of pig, dog, cat, horse, sheep, monkey and bovine sera were prepared using method 10 of Cohn, Gurd, Surgenor, Barnes, Brown, Derouaux, Gillespie, Kahnt, Lever, Liu, Mittelman, Mouton, Schmid and Uroma (1950). Corresponding fractions of rabbit, rat and guinea-pig sera were prepared by the modification of method 10 introduced by Goldstein and Anderson (1957).

(2) *Preparation of fluorescent amines*

Fluorescein amine isomers were prepared by the method of Coons and Kaplan (1950).

Nitrorhodamine was prepared by heating equal parts by weight of *m*-NN diethylaminophenol and 4-nitrophthalic acid at 170–174° C. until the mass was dry (3–4 hours). After cooling, the melt was chipped from the beaker, ground to a powder and allowed to stand overnight in 1 N ammonium hydroxide. After filtering and drying the resultant crude nitrorhodamine was suspended in absolute ethanol and reduced to the amine with hydrogen at room temperature and atmospheric pressure in the presence of Raney nickel catalyst.

The Raney nickel was removed by centrifugation and washed with a small volume of ethanol. Addition of 4 volumes of water to the ethanolic solution caused precipitation of the amine. The precipitate was found to be too fine to filter by ordinary methods and slow to separate on centrifugation. Therefore it was extracted with a small volume of ethylacetate and crystallized from the solvent.

Fluorescein amine and rhodamine amine were converted to the corresponding isocyanates by treatment with phosgene under anhydrous conditions and conjugated with the appropriate globulin fractions by the method of Coons and Kaplan (1950). Following conjugation dioxane, acetone and excess free fluorescein or rhodamine derivatives were removed by ethyl acetate extraction (Dineen and Ada, 1957) prior to use.

Sections of tissue, where practicable, were taken from the margins of various

tumours, prepared by a method previously described (Louis, 1957*a*) and were treated with the appropriate stain for 10 minutes at room temperature. The sections were washed in three changes of phosphate buffered saline for 10 minutes prior to examination. For microscopy a Leitz fluorescence microscope fitted with two BG 4 mm. filters between light source and condenser and a Wratten G 15 gelatin filter in the ocular was used.

RESULTS

Sections of each of a large series of tumours, both innocent and malignant, were individually treated with the above enumerated stains. In all cases identical staining affinities were found for a particular tissue regardless of which animal provided the globulin used or whether a fluorescein or rhodamine conjugate was employed to enable visualization of the globulin; the results are presented in tabular form.

The results of the series of human tumours investigated are shown in Table I and those of animals in Table II.

TABLE I

Results obtained by staining human tissues with conjugated globulins. Both fluorescein and rhodamine were used but, since there was no appreciable difference between the two regarding intensity and type of staining, these were not given separately. In all cases the results were comparable, that is non-neoplastic tissue stained and fluoresced well but malignant neoplastic tissue did not; nevertheless the numbers and types of cases examined are set out in detail. The globulins of all the sera (other than rabbit) were examined in groups so that the figures (number of cases) apply to all the genera cited. (+) = positive staining, (−) = absence of staining.

Human material examined		Results obtained with different globulins	
Tissue	Condition	Normal rabbit	Rat, guinea-pig, pig, mouse, horse, cattle, sheep, man, monkey, dog, cat, fowl
Skin	Normal epithelium	(+)28	(+)7
	Molluscum pseudo-carcinomatosum	(+) 6	(+)3
	Squamous-cell carcinoma	(−) 8	(−)4
Breast	Hyperplasia	(+)54	(+)6
	Fibroadenoma	(+) 7	(+)3
	Carcinoma	(−)26	(−)6
Stomach	Normal mucosa	(+) 8	(+)5
	Carcinoma	(−) 5	(−)3
Colon	Normal mucosa	(+)32	(+)3
	Polyp	(+) 2	(+)2
	Carcinoma	(−)28	(−)3
Lung	Bronchial mucosa	(+)12	(+)3
	Carcinoma	(−) 9	(−)3
Thyroid	Normal	(+)26	(+)5
	Colloid goitre	(+)18	(+)5
	Foetal adenoma	(+) 8	(+)3
Lymph node	Normal lymphoid tissue	(+)10	(+)3
	Secondary melanoma	(−) 1	(−)1
	Secondary carcinoma, breast	(−) 5	(−)2

Representative examples of these cases are illustrated in Fig. 1–9. These have been chosen, at random, from a large series of specimens which have been examined and photographed. It can be seen that the innocent tissue in these illustrations fluoresces whilst the neoplastic tissue does not fluoresce. In the so-called innocent tumours both the normal and pathological tissues fluoresce. Preneoplastic rat liver resulting from the feeding of 4-dimethylaminoazobenzene displayed islands of morphologically normal parenchymal cells with decreased

---

EXPLANATION OF PLATES

FIG. 1.—Photomicrograph (with ultra-violet light) of a frozen section of margin of a *rat* hepatoma induced by feeding 3′-methyl-4-dimethylaminoazobenzene showing fluorescence of cytoplasm of normal cells and absence of fluorescence of cytoplasm of neoplastic cells. Stain—fluorescein-fowl globulin complex.

FIG. 2.—Photomicrograph showing the same area as in Fig. 1 stained with haematoxylin and eosin; the structure of the non-fluorescing area is demonstrated.

FIG. 3.—Photomicrograph (with ultra-violet light) of a frozen section of the margin of a *rat* cholangioma induced by feeding 3′-methyl-4-dimethylaminoazobenzene and showing fluorescence of cytoplasm of normal cells and absence of fluorescence of neoplastic cells. Stain—fluorescein-human globulin complex.

FIG. 4.—Photomicrograph showing the same area as in Fig. 3 stained with haematoxylin and eosin; the glandular and neoplastic character of the non-fluorescing area is demonstrated.

FIG. 5.—Photomicrograph of a frozen section of *rat* liver after feeding 4-dimethylaminoazobenzene for 6 weeks showing an island of parenchymal cells which have lost their affinity for the stain. Stain—rhodamine-rabbit globulin complex.

FIG. 6.—Photomicrograph of the same section as in Fig. 5 stained with haematoxylin and eosin showing the morphology of the non-staining cells.

FIG. 7.—Photomicrograph of a frozen section of the margin of a spheroidal cell carcinoma of the stomach (*man*) showing fluorescence of the cytoplasm of normal cells and absence of fluorescence of the neoplastic cells. Stain—fluorescein-dog globulin complex.

FIG. 8.—Photomicrograph of the same area as in Fig. 7 stained with haematoxylin and eosin; the structure of the non-staining area is that of a typical spheroidal cell carcinoma.

FIG. 9.—Photomicrograph of a frozen section prepared from a mixed salivary tumour (*man*) showing fluorescence of epithelial cells in cellular areas as well as those in the mucoid areas. Stain—fluorescein-monkey (rhesus) globulin complex.

FIG. 10.—Photomicrograph of the same area as in Fig. 9 stained with haematoxylin and eosin to show the morphological characters of the fluorescing cells. These appearances indicate this growth (at this stage) corresponds with the non-neoplastic rather than the neoplastic conditions.

FIG. 11.—Photomicrograph of a frozen section prepared from a *human* lymph node containing secondary melanomatous deposits showing lack of fluorescence in the secondary deposits. Stain—fluorescein-mouse globulin complex.

FIG. 12.—Photomicrograph of same area as in Fig. 11 stained with haematoxylin and eosin showing that both melanotic and amelanotic tumour areas do not fluoresce. The fluorescing area in Fig. 11 is a collection of reticulo-endothelial cells.

FIG. 13.—Photomicrograph of a frozen section of a transplanted carcinoma of the breast in a *mouse* showing fluorescence of adjacent skin but absence of fluorescence in the subjacent area. Stain—fluorescein bovine globulin complex.

FIG. 14.—Photomicrograph of the same area as in Fig. 13 stained with haematoxylin and eosin showing the structure of the non-fluorescing area to be that of a typical epithelial (carcinomatous) tumour.

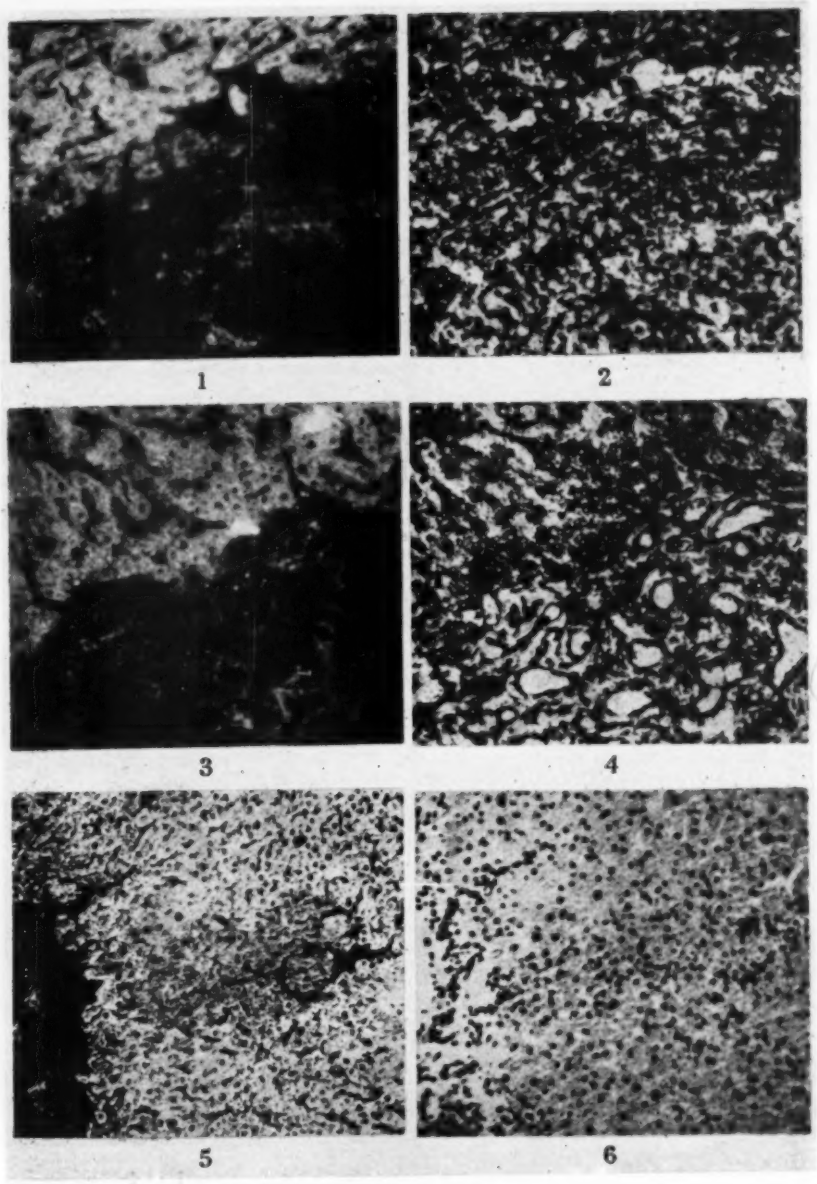
FIG. 15.—Photomicrograph of a blood film taken from a *mouse* suffering from chronic lymphatic leukaemia showing fluorescence of leucocytes and absence of fluorescence of erythrocytes. Stain—fluorescein-sheep globulin complex.

FIG. 16.—Photomicrograph of same area as in Fig. 15 stained by Leishman's method, showing that all the leucocytes fluoresce.

FIG. 17.—Photomicrograph of frozen section of a fibroadenoma of the *human* breast showing fluorescence of the epithelial cells lining the ducts. Stain—fluorescein-horse globulin complex.

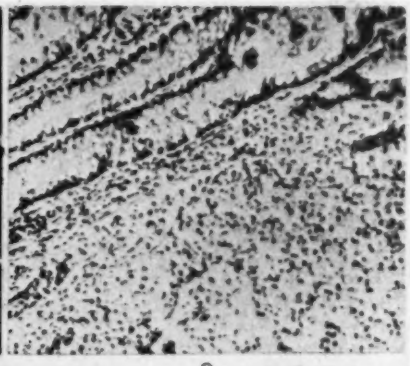
FIG. 18.—Photomicrograph of same area as in Fig. 17 stained with haematoxylin and eosin.

All magnifications × 120.

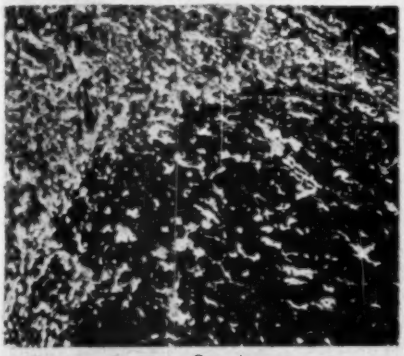


King, Hughes, and Louis.

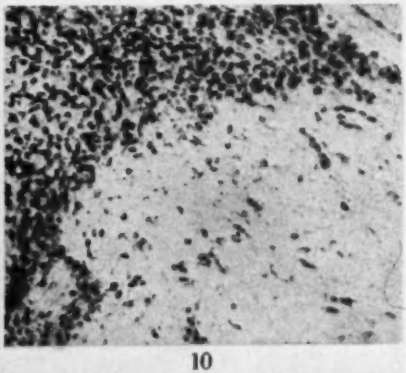
7



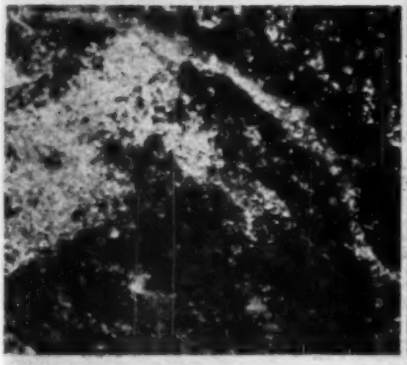
8



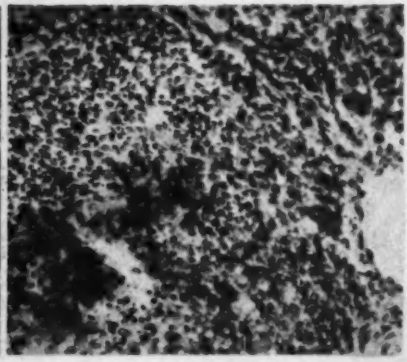
9



10

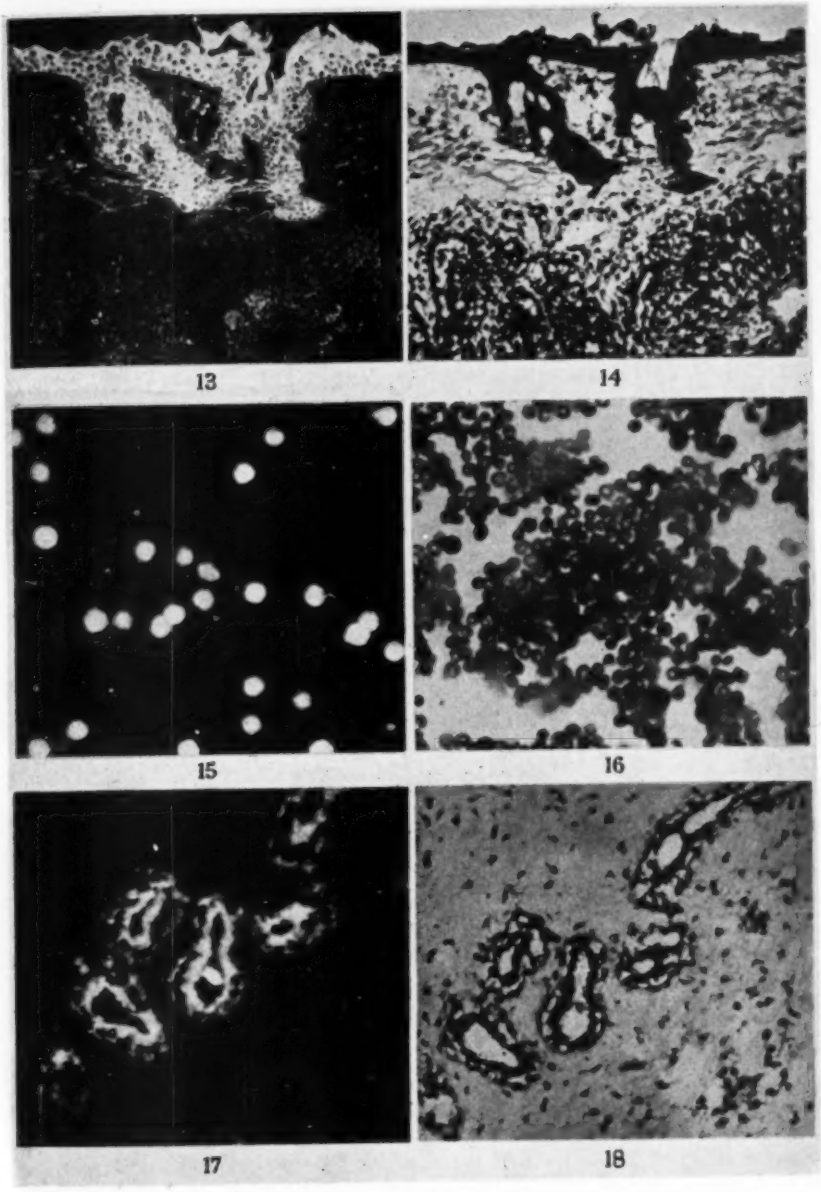


11



12

King, Hughes and Louis.



13 14

15 16

17 18

King, Hughes and Louis.

Table II

Results obtained by staining various tissues (normal and tumour) with different globulin complexes. Both fluorescein and rhodamine were used (see Table I). Although exceptions to the staining characters described were not found, the results are set out in detail to indicate the actual extent of the investigation to date. Figures give number of cases examined; + = positive staining, − = absence of staining.

Conditon	Results obtained with globulins from different species (No. of cases examined)												
	Rabbit	G.-pig	Rat	Mouse	Pig	Horse	Cattle	Sheep	Man	Mon-key	Dog	Cat	Fowl
Skin (normal)	+10	+3	+3	+5	+2	+2	+2	+2	+3	+3	+2	+2	+3
Liver (normal)	+6	+1	+1	+1	+1	+2	+2	+1	+1	+1	+1	+1	+2
W.B.C. (normal)	+56	+5	+5	+6	+3	+2	+3	+2	+4	+2	+2	+2	+7
R.B.C. (normal)	−56	−5	−5	−6	−2	−2	−3	−2	−4	−2	−2	−2	−7
Spont. ca. breast	−1	−1	−1	−1	−7	−1	−1	−1	−1	−1	−1	−1	−1
Transpl. ca. breast	−7	−2	−2	−4	−1	−1	−2	−1	−2	−1	−1	−1	−2
W.B.C. (ac. leukaemia)	−28	−3	−3	−4	−2	−1	−2	−1	−2	−1	−1	−1	−4
Liver (normal)	+48	+1	+3	+1	+1	+1	+2	+1	+13	+1	+1	+1	+3
Hepatoma	−6	−2	−2	−2	−2	−2	−2	−2	−6	−2	−2	−2	−2
Cholangioma	−4	−2	−2	−2	−2	−2	−2	−2	−4	−2	−2	−2	−2
Ca. breast	−3	−1	−3	−1	−1	−1	−2	−1	−1	−1	−1	−1	−2
Liver (normal)	+2	+1	+1	+2	+1	+2	+2	+1	+2	+1	+1	+1	+2
W.B.C. (normal)	+3	+1	+1	+2	+2	+1	+2	+1	+2	+1	+1	+1	+2
R.B.C. (nuclei)	+3	+1	+1	+2	+2	+1	+2	+1	+2	+1	+1	+1	+2
Liver (normal)	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1
Second ca. liver	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1
Ca. adrenal	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1

affinities for both fluorescein and rhodamine conjugates of the various globulins (Fig. 5, 6).

No significant difference in intensity of staining was found with the different globulins. Modifications of technique were not employed, that is to say, the same staining times under strictly comparable conditions were used in all cases. The degree of fluorescence both as observed by the eye and as recorded on the photographic plate was of the same, or at least comparable, degree in all cases.

The intensity of fluorescence observed in these studies is not directly comparable with that found when dealing with specific fluorescent antibody-antigen reactions. It is a more general type of fluorescence classed as non-specific by Coons and Kaplan (1950) so that from these results it may be stated that normal as opposed to malignant tissue has an affinity for the component of the stain giving, immunologically speaking, non-specific fluorescence. Repeated absorption of these sera with acetone-dried mouse liver particles removes this type of fluorescence staining completely. As with any other form of staining with tissues, certain conditions have to be met to give satisfactory cytological differentiation. In the present case unconjugated fluorescein derivatives must be removed. This has been accomplished by ethyl acetate extraction of the sera since this method preserves the active component of the stain.

DISCUSSION

In this investigation we have found that the staining characteristics of conjugates prepared from several globulins have been identical, regardless of the species of origin of the globulin. The result is thus independent either of any species variations in the globulins tested or in the means of serum fractionation employed in obtaining the required fractions.

It is known that the globulins of different orders differ considerably; for example, those of the larger ungulates, such as the horse and cattle, have a much higher molecular weight than the globulins of some of the other animals. Thus it might have been expected that these would act differently but, in fact, both from the point of view of their formation of fluorescein and rhodamine conjugates and also their reaction with various types of tissues, they were essentially similar to those of other animals. This, therefore, indicates that the characteristics of the stain complex are due to features other than the molecular weight of the globulin or any special peculiarity of its intramolecular structure.

In the early stages of the investigation, rabbits were injected with homogenates of tissues of various kinds from various animals. When the question was raised as to the significance of the antigen-antibody reaction, some of the presumable "specific antibody globulin" for one tissue was used for some other, for example, globulin from a rabbit which had been injected with colon mucosa as the antigen was used for breast tissue. It was found that it produced as distinctive differentiating staining as occurred when the globulin from an animal injected with the appropriate tissue was employed.

Various methods of fractionation of the proteins of the serum were employed but no significant difference was found in the staining of these various protein conjugates. This would suggest that the staining phenomenon described is not peculiar to one particular type of protein and that the effect might be produced by a much wider range of proteins than was previously expected. This will be a matter for future study but, for the present, it is apparent that not only the γ-globulins but the immediately related globulins also act in a similar manner. This further supports the suggestion that the phenomenon is not a serological antigen-antibody reaction but rather some physico-chemical interaction due to the physical properties (possibly those of the electric charge) of the serum proteins.

Sorof and Cohen (1951) have shown, in a variety of tumours, that there is a significant reduction in the proportion of the more basic cytoplasmic proteins. These proteins, because of their extreme basicity, could be expected to form complexes with globulins selectively over a wide range of pH. Such differential staining, as has been observed, is therefore not unexpected in view of these electrophoretic findings.

The results obtained, moreover, are in entire agreement with such a view. The γ-globulins form a relatively homogeneous class of protein and if anion-cation complex formation is involved they could be expected to behave similarly because of similarities in their isoelectric points and electric charges. Of special significance are the observations that the homologous globulin is equally efficacious in obtaining the staining as any heterologous globulin. Thus human tissues stain equally well with human γ-globulin conjugates as with those prepared from any other animal.

Since similar differential staining could be achieved with either fluorescein or rhodamine conjugates the individuality of the dye moiety could not be implicated. Thus it can be seen that the γ-globulins as a class of proteins are all equally suitable for demonstrating this staining reaction.

There are a few points of special technical importance. When tissues are exposed to ultra-violet light some of them will demonstrate a fluorescence which is peculiar to them themselves. This is quite different and distinct from that of the stains which have been used but, nevertheless, requires to be specially differentiated. This autofluorescence occurs particularly in elastic tissue and in keratin and it has, here, a characteristic yellow and bluish-white colour respectively. This is quite distinct from the green colour of the fluorescein or the orange of the rhodamine but, in any case, can be readily differentiated by first examining the section under the ultra-violet light without any staining.

We have found that not all normal cells stain by this method. The cells which do stain are normal epithelial cells and some phagocytic cells. Neoplastic cells, whatever their origin, do not stain. This is clearly a property of the neoplastic cell and is not a function of the amount of the cytoplasm in the cell. Normal connective tissue cells, possibly by view of decreased cytoplasm, do not stain. It would seem that the intercellular substance does not contain the basic protein complex which is the characteristic of the cytoplasm of the epithelial cells.

This investigation is still in its early stages and much information concerning the types and characteristics of proteins of various kinds of cells and of the intercellular substance of the various connective tissues is required before far-reaching conclusions can be drawn. However, for the time being, it seems clear that most epithelial cells contain, amongst the variety of the cytoplasmic proteins, a basic protein which, from the point of view of the present study, is the focal point of this staining reaction. This protein appears to be present also in some of the connective tissue cells, particularly some of the wandering cells and endothelial cells which possess a considerable amount of cytoplasm in their structure.

While many characteristics of tumour cells have been described, none has been found to be peculiar *per se* to the neoplastic state. The pathologist has always had to resort to the general architecture of the tissue for a true assessment of the condition. Here, however, we have a clearly defined feature which distinguishes sharply the tumour cell from its normal counterpart. At first it seemed as if this was a characteristic peculiar to certain chemical carcinogen-induced neoplasms and that the phenomenon was a complex biological antigen-antibody reaction. The multiplicity of the globulins which show the characteristic staining reaction (and particularly the applicability of the homologous globulins in this regard) demonstrates the relatively simple physico-chemical interrelation between proteins of different electrical charges which underly this phenomenon.

The ability of this protein of the normal cells to form complexes with fluorescein-globulin conjugates selectively (and thus to be stained selectively) and conversely the inability of malignant cells, which lack the protein, to demonstrate any such staining appears to be a feature by which individual neoplastic cells may be recognized. Not only is this a matter of prime importance, as a method of investigation, in the recognition of malignant tissue in cases where its normal recognition is difficult but it emphasizes an important distinction between the malignant and the normal cell and this has already proved to be a crucial difference which has far-reaching implications in relation to several aspects of basic cancer research.

SUMMARY

Following injection of rabbits with homogenates of rat tissue, a rabbit γ-globulin was shown to combine with normal rat tissue cells but not with tumour cells of the same organ. This was demonstrated visually by the use of a globulin-fluorescein complex which fluoresced in ultra-violet light.

Although at first regarded as an antigen-antibody reaction it was shown, by the demonstration of the same characteristics when normal rabbit globulin was used instead of that from an injected rabbit, to be a protein interaction of no serological significance.

This feature was examined first in the case of tumours produced in animals following the administration of chemical carcinogens but was applied subsequently to naturally occurring tumours of both animals and man.

The method has now been employed also using globulins from normal mouse, rat, guinea-pig, horse, cow, sheep, pig, dog, cat, monkey, man and fowl. In all cases the results obtained were so similar as to be indistinguishable in degree and kind from those observed with the rabbit sera.

The method of production of the globulin was different in different cases and in the rabbit more than one method was employed. No difference was observed in the results.

The stains were prepared by conjugating the globulins with both fluorescein and rhodamine without any significant difference (other than colour) being seen in the staining effects.

It appears, therefore, that the results observed (within the range of animals examined) are due to physico-chemical characteristics of globulin irrespective of the species from which it is obtained. The phenomenon of staining of normal tissue and non-staining of malignant tissue, therefore, by the use of globulin-fluorescein conjugates, is a physico-chemical interaction depending on peculiarities other than those of a serological character.

We are indebted to the Commonwealth Serum Laboratories for several of the serum fractions; technical assistance with the conjugation of globulins provided by Staff Members of the Defence Research Laboratories, Melbourne, is gratefully acknowledged; and thanks are tendered to Mr. W. R. Mackay, Assistant Pharmacist, Royal Melbourne Hospital, for cheerful aid in various stages of the work. This work was supported by the Anti-Cancer Council of Victoria.

REFERENCES

Cohn, E. J., Strong, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J. N., Melin, M. and Taylor, H. L.—(1946) *J. Amer. chem. Soc.*, **68**, 459.

Idem, Gurd, F. R. N., Surgenor, D. M., Barnes, B. A., Brown, R. K., Derouaux, G., Gillespie, J. M., Kahnt, F. W., Lever, W. F., Liu, C. H., Mittelman, D., Mouton, R. F., Schmid, K. and Uroma, E.—(1950) *Ibid.*, **72**, 465.

Coons, A. H. and Kaplan, M. H.—(1950) *J. exp. Med.*, **91**, 1.

Dineen, J. K. and Ada, G. L.—(1957) *Nature, Lond.*, **180**, 1284.

Goldstein, J. and Anderson, J. W.—(1957) *J. biol. Chem.*, **224**, 775.

Hughes, P. E., Louis, C. J., Dineen, J. K. and Spector, W. G.—(1957) *Nature, Lond.*, **180**, 289.

Idem.—(1957) *Aust. N.Z. J. Surg.*, **26**, 302.

LOUIS, C. J.—(1957*a*) *Stain Tech.*, **32**, 279.
Idem.—(1957*b*) *Aust. N.Z. J. Surg.*, **27**, 146.
Idem.—(1957*c*) *Aust. Ann. Med.*, **6**, 300.
MILLER, E. C. AND MILLER, J. A.—(1947) *Cancer Res.*, **7**, 468.
SOROF, S. AND COHEN, P. P.—(1951) *Ibid.*, **11**, 376.
WEILER, E.—(1952) *Z. Naturf.*, **7b**, 324.
Idem.—(1956) *Ibid.*, **11b**, 31.

RETICULIN PATTERNS IN THE DIAGNOSIS OF CARCINOMAS AND SARCOMAS

D. H. MACKENZIE

From the Department of Morbid Anatomy, Westminster Hospital, London

Received for publication November 20, 1957

RETICULIN has been studied for just over a hundred years. Since Kölliker (1852) described the reticular tissue in Peyer's patches of the intestine, a considerable literature has accumulated. Much of this has been devoted to descriptions of reticulin patterns in normal tissues and, above all, to the nature of the reticular substance. Differences of opinion still exist as to the relationship between reticulin and collagen (Brewer, 1957), and indeed on the nature of reticulin itself, which may prove to be of more than one type (Windrum, Kent and Eastoe, 1955; Jackson and Williams, 1956).

Since 1900 a number of workers have studied the distribution of reticulin in tumours, and attempts have been made to utilise reticulin patterns as aids to diagnosis and, in particular, to distinguish carcinomas from sarcomas in difficult cases. Much of this work is to be found in the German literature and most of the contributors in English have only been concerned with the reticulin of individual neoplasms without reference to the general diagnostic possibilities. It is with this last aspect of reticulin that this communication is concerned. As used here, the word reticulin refers to those argyrophilic fibres which are found in varying amounts and with variable distribution within the substance of carcinomas and sarcomas. The distribution of reticulin in tumours of lymphoid tissue will be discussed in a separate communication.

There has been general agreement that the reticulin patterns in carcinomas are different from those normally seen in sarcomas. Controversy has arisen as to whether these differences are sufficiently constant to be valuable in diagnosis and, above all, whether the patterns are maintained in cellular and anaplastic neoplasms which form the group where diagnosis is frequently most difficult. White (1900), Niosi (1915), Edelmann (1925) and, in particular Ascher (1934) carried out extensive investigations into the reticulin patterns of malignant tumours and came to the conclusion that the method was of definite value in distinguishing carcinomas from sarcomas. In the former they found that the reticulin fibrils tended to be arranged in an alveolar fashion surrounding groups of cells (Fig. 1, 2, and 3). Sometimes the groups were large and gave the impression of sheets of tumour cells with only perivascular reticulin. In sarcomas, however, they noted a network of fine fibrils often closely related to individual tumour cells and forming an intercellular supporting meshwork for them (Fig. 4, 5, and 6). The alveolar pattern found in carcinomas was not seen in the connective tissue group.

Reference must now be made to the very admirable M.D. thesis submitted at the University of St. Andrews in 1937 by the late G. R. Tudhope. As far as the writer has been able to determine this work was never published. He described

the historical background to the study of carcinomas and sarcomas and discussed at length the stromatic architecture of many tumours in terms of the collagenous stroma and the reticular stroma. He considered both the nature and origin of reticulin and described its distribution in many cases which he had personally studied. He was particularly concerned with its value in diagnosis and he came to the conclusion that, even in the most anaplastic and cellular tumours, a study of the reticulin pattern was often of value in distinguishing diffuse carcinomas from sarcomas. He believed this to be true both of primary tumours and of metastases. In carcinomas he described the reticulin as having a peri-alveolar distribution around groups of tumour cells whereas in sarcomas intercellular fibrils occurred, and the alveolar pattern was lacking. He stressed that, in some very cellular carcinomas, reticulin was scarce and the alveolar pattern was lacking. However, even in those neoplasms, there was no reticulin in the midst of cell masses, or closely associated with individual cells. During these years other workers studied reticulin patterns but were not convinced of their constancy or of their diagnostic value. Speciale (1924) saw no essential difference in reticulin patterns in epithelial and mesodermal neoplasms but some of the tumours of lung, ovary and adrenal selected as examples of sarcomas cannot be accepted. Cohn (1926) believed that a degree of anaplasia could be reached when both carcinomas and sarcomas lost their typical patterns which were therefore unreliable in those very cases when help was needed most. Willis (1953) forcibly expresses a similar view as follows :

> " It is time, too, for pathologists to recognise that study of the fine relationships of stromal collagen or reticulin to the cells of anaplastic tumours of doubtful nature will not enable carcinomas, sarcomas and other tumours to be distinguished."

He continues :

> " My opinion after considerable experience of special stains for connective tissue and reticulin is that, while these often do delineate fairly characteristic patterns in typical specimens of particular classes of tumours, they are of no value in identifying tumours of unusual structure or uncertain nature."

In addition to papers concerned with the broad distinction between carcinomas and sarcomas there have been a number of other communications on reticulin patterns. Foot and Day (1925) stated that the absence of reticulin in neuroblastomas distinguished them from lymphosarcomas. Traut, Kuden and Cadden, (1939) stated that a study of reticulin distribution enabled granulosa cell tumours to be distinguished from those composed of theca cells. They found that theca cells were surrounded individually by argyrophil fibres whereas granulosa cells and lutein cells were either not enclosed at all or were enclosed in groups. Hsu (1940) described a number of intracranial tumours apparently arising from the meninges, and maintained that the presence of fine reticulin fibrils proved their connective tissue origin. A similar type of case was described by Tuthill and Meredith (1943). Gouyou (1946), in the course of his study of reticulin in seminomas, found that the reticulin pattern was of value in distinguishing necrotic tumours which contained reticulin from gummata. Valls, Musculo and Schajowicz (1952) claimed that the amount of reticulin in reticulum cell sarcomas of bone was greater than that observed in Ewing's tumour and this has been the writer's experience (Lumb and Mackenzie, 1956).

It is clear that there is no unanimity of opinion regarding the value of silver impregnation in the diagnosis of malignant tumours and a further investigation seemed worthwhile. In the present study the reticulin patterns have been observed in three hundred primary and secondary tumours. These have been made up of one hundred and sixty-four carcinomas and one hundred and thirty-six sarcomas of various kinds. Reticulum cell sarcomas were examined as these provide a differential diagnosis from anaplastic carcinomas. Attention has been focused mainly on the more anaplastic tumours and these have only been accepted for study where clinical, histological and often autopsy studies have placed the diagnosis beyond reasonable doubt. All sections were stained with haematoxylin and eosin and by Gomori's silver impregnation technique. Table I shows the number of tumours studied and their respective types.

TABLE I

Carcinomas—	
Anaplastic squamous carcinomas	48
Metastatic carcinomas	40
Miscellaneous carcinomas	35
Oat-cell carcinomas of the lung	20
Adenocarcinomas of intestinal wall and lungs	15
Carcinomas of the kidney	6
	164
Sarcomas—	
Fibrosarcomas	28
Osteogenic sarcomas	27
Synovial sarcomas	20
Metastatic sarcomas	20
Reticulin-cell sarcomas	18
Miscellaneous sarcomas of uncertain origin	11
Leiomyosarcomas	5
Liposarcomas	5
Rhabdomyosarcomas	2
	136

Tables II and III show the carcinomas and sarcomas considered collectively but divided into those cases where plenty of material was available for study and those where only biopsy fragments were seen.

EXPLANATION OF PLATES.

FIG. 1.—Alveolar pattern in poorly differentiated adenocarcinoma of stomach. Silver impregnation. × 190.

FIG. 2.—Alveolar pattern in metastatic carcinoma of breast in axillary node. Silver impregnation. × 190.

FIG. 3.—Alveolar pattern in hypernephroma. Silver impregnation. × 120.

FIG. 4.—Fibrillary pattern in fibrosarcoma. Silver impregnation. × 175.

FIG. 5.—Fibrillary pattern in reticulin-cell sarcoma. Silver impregnation. × 175.

FIG. 6.—Fibrillary pattern in cerebral metastasis from osteogenic sarcoma. Silver impregnation. × 180.

FIG. 7.—Mixed pattern in synovial sarcoma. Silver impregnation. × 100.

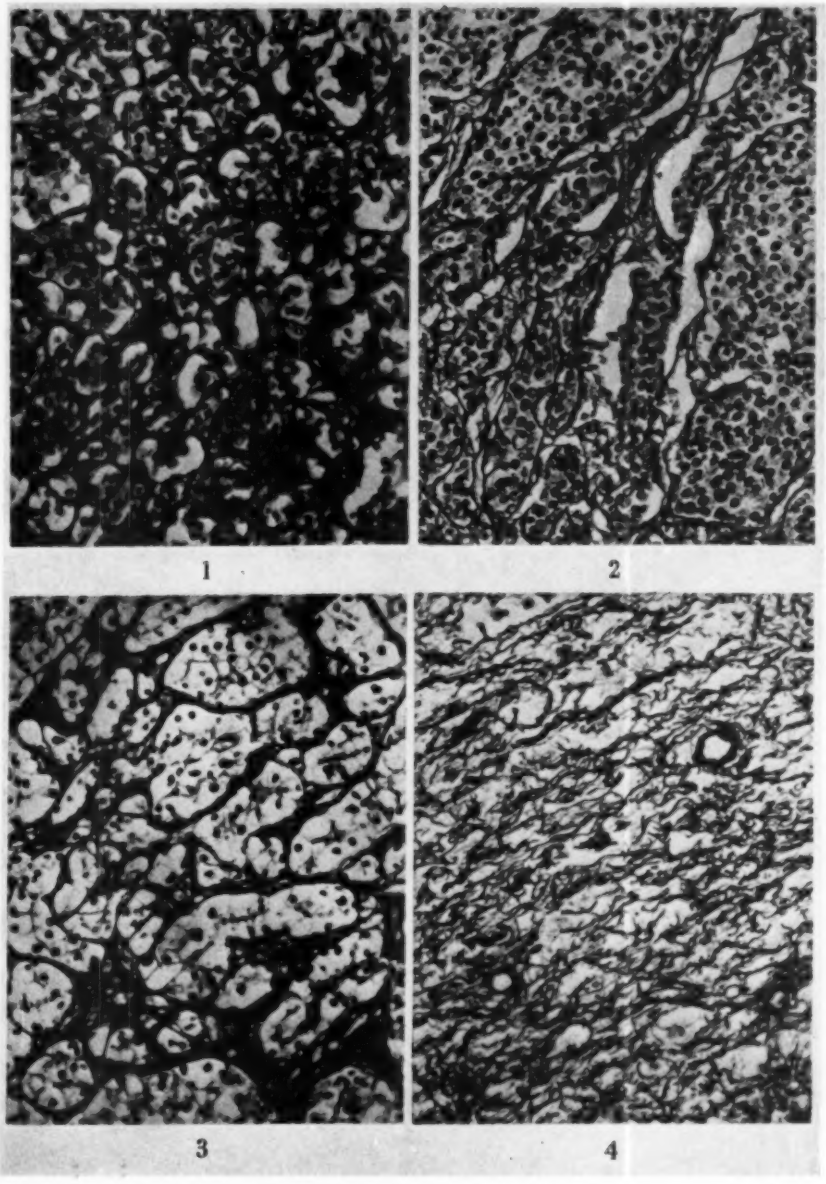
FIG. 8.—Alveolar pattern in synovial sarcoma. Silver impregnation. × 145.

FIG. 9.—Case I. Alveolar pattern in anaplastic metastasis in lymph-node from carcinoma of lung. Silver impregnation. × 280.

FIG. 10.—Case II. Absence of intercellular reticulin and alveolar pattern in anaplastic carcinoma. Silver impregnation. × 145.

FIG. 11.—Case III. Fibrillary pattern in sarcoma of uterus. Silver impregnation. × 180.

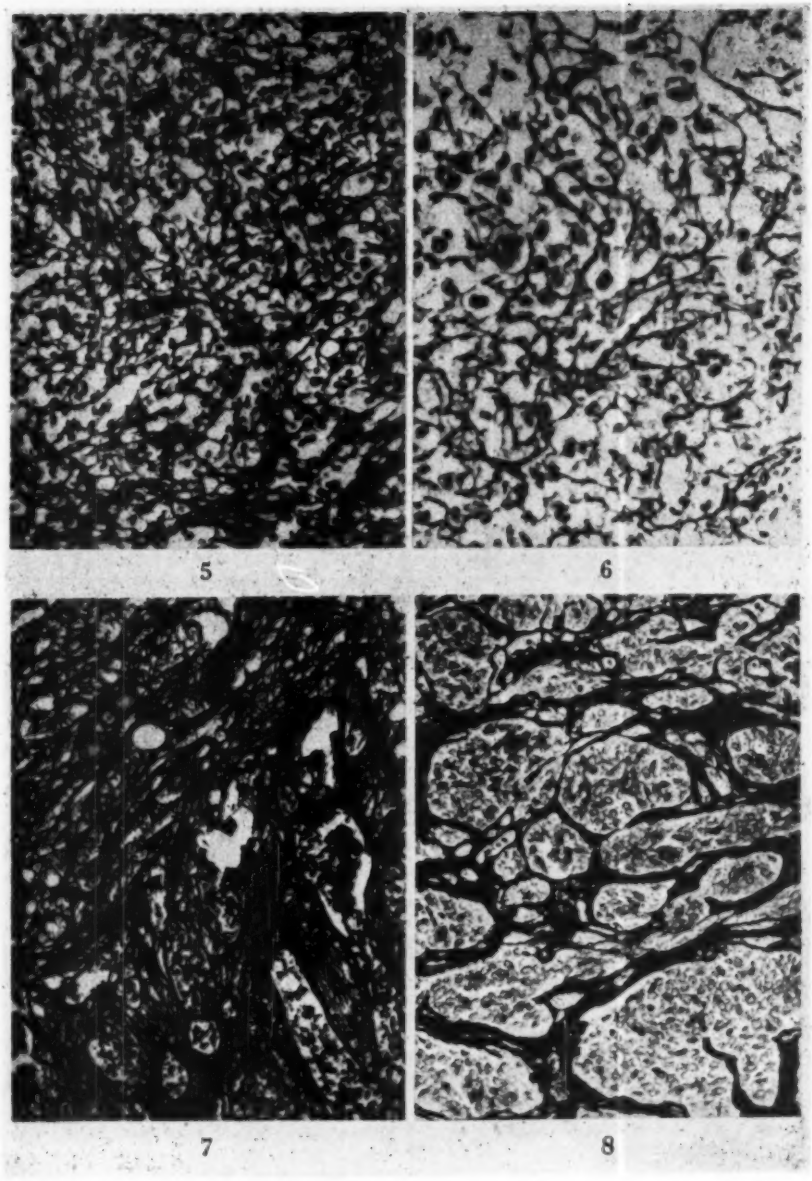
FIG. 12.—Misleading alveolar pattern in fibrosarcoma. Silver impregnation. × 90.



1 2

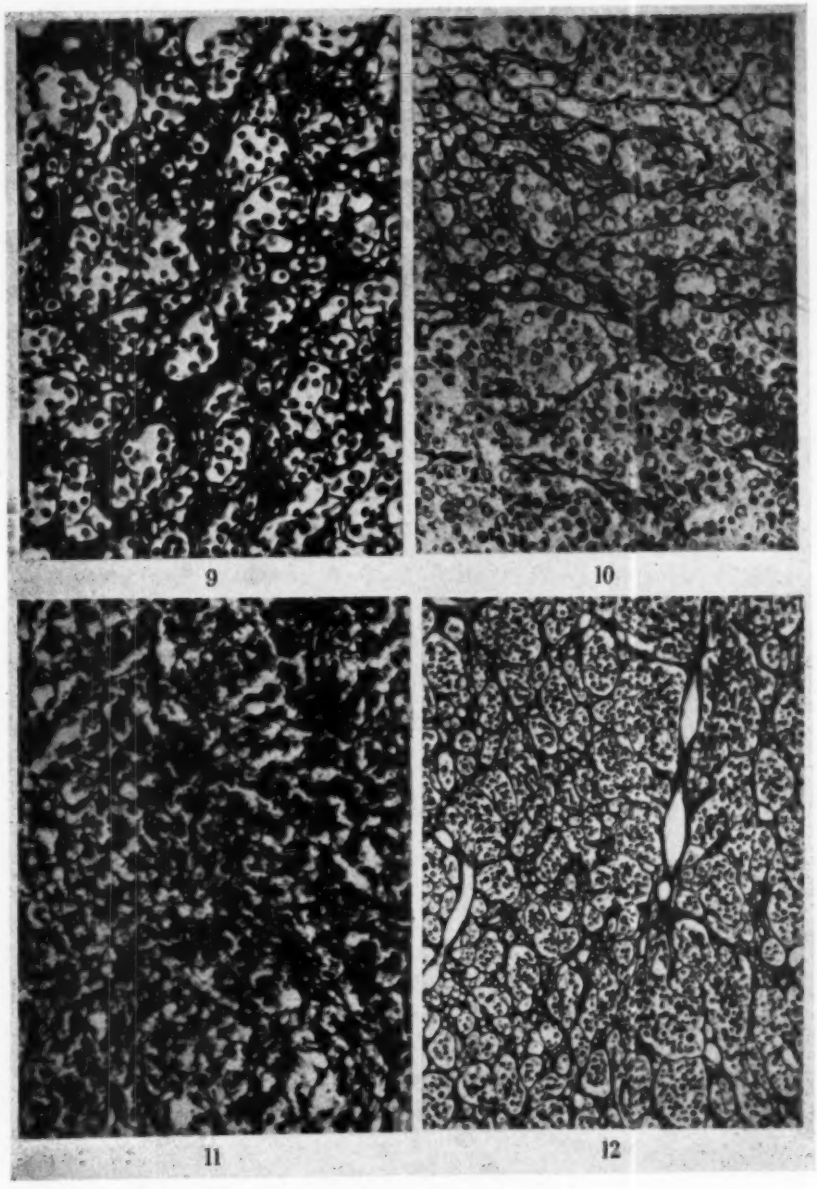
3 4

Mackenzie.



5 6

7 8



9 10

11 12

TABLE II.—*Adequate Tissue Available*

	Typical carcinomatous pattern	Predominantly carcinomatous pattern	Inconsistent or misleading
Primary tumours	52	32	6
Metastases	20	3	0

	Typical sarcomatous pattern	Predominantly sarcomatous pattern	Inconsistent or misleading
Primary tumours	50	17	7
Metastases	13	3	1

TABLE III.—*Fragments Only Available*

	Typical carcinomatous pattern	Mixed pattern
Primary tumours	20	14
Metastases	11	6

	Typical sarcomatous pattern	Mixed pattern
Primary tumours	15	7
Metastases	2	1

In Tables II and III synovial sarcomas have been omitted since they have characteristic and variable reticulin patterns. A number of writers including Haagersen and Stout (1944), Bennett (1947), Tillotson, McDonald and Janes (1951), Wright (1952), and others have described their histology in detail and have pointed out that two components, the fibrosarcomatous and the pseudo-epithelial, often co-exist. This fact is reflected in the reticulin patterns and, of the twenty cases studied in this series, eight had a mixed pattern (Fig. 7), seven a carcinomatous one, and five a purely sarcomatous fibrillary pattern. Pseudo-epithelial differentiation produces a carcinomatous alveolar distribution of reticulin (Fig. 8). Silver impregnation was not found to be of value in distinguishing other forms of sarcomas from one another. Bone forming osteogenic sarcomas, liposarcomas with a high fat content and fibrosarcomas with extreme myxomatous degeneration often gave unsatisfactory reticulin preparations but the fibrillary picture was still apparent. Fibroblastic tumours such as the dermatofibrosarcoma and the desmoid tumour conformed to the sarcomatous pattern in the few cases examined.

In Table II the importance of those cases showing only a predominating pattern lies in the fact that a biopsy might well have included only the atypical areas. With metastatic tumours in lymph-nodes information can be gained not only from the pattern of the tumour reticulin itself but also, by outlining the sinuses, it is often possible to see the exact location of abnormal cells. Silver impregnation is of considerable help in distinguishing a lymph-node destroyed by a reticulin-cell sarcoma from one destroyed by an anaplastic carcinoma.

Fig. 9, 10, and 11 illustrate three cases where the method was of value. In Case I an axillary lymph-node was found to be replaced by a pleomorphic and

anaplastic tumour and a diagnosis of Hodgkin's disease was suggested. At a subsequent autopsy a primary carcinoma of the lung was found. Fig. 9 shows the original lymph-node stained for reticulin. In Case II the diagnosis lay between an anaplastic carcinoma and a reticulum-cell sarcoma. Fig. 10 shows the carcinomatous reticulin pattern and this diagnosis was borne out by the progress of the disease. Fig. 11 illustrates the fibrillary pattern in a uterine tumour biopsy. Originally diagnosed as an anaplastic carcinoma, multiple sections from the main tumour mass revealed a pleomorphic sarcoma.

DISCUSSION

This study of three hundred primary and secondary carcinomas and sarcomas suggests that a study of reticulin patterns by a silver impregnation technique can be a valuable aid in the diagnosis of primary and secondary tumours of uncertain origin. This value increases if the following factors are fully appreciated.

I. Adequate material

Examination of Tables II and III, shows that mixed misleading patterns are often encountered when only fragments are available for study. Fig. 12 shows an isolated area in what was otherwise a typical fibrosarcoma. This area, though small, was larger than a biopsy fragment might have been. It seems inadvisable therefore to place reliance on reticulin patterns unless adequate material is available. Fragments are also liable to include artefacts due to crushing and diathermy.

II. The hazard of pre-existent reticulin

Tudhope (1937) stated that reticulin patterns, particularly in metastases, should always be examined in the heart of the tumour and not near the invasive margin. He believed that, at the margin, the tumour reticulin was intimately mixed with the pre-existing reticulin of the part being invaded and that very misleading pictures could result. He also suggested that, at times, the reticulin content of an invaded organ could alter at points ahead of the actual tumour cells, which made it doubly desirable to examine the pattern in well-established areas of growth. This is clearly a wise precaution though, in the writer's experience, reticulin patterns are soon imposed and may be quite typical even near the margin. Occasionally very small groups of metastatic sarcoma cells may appear surrounded by the reticulin of the invaded part giving a false impression of an alveolar pattern.

III. Chance distribution

In certain instances, particularly when reticulin is abundant, reticulin fibres may link up and give the impression of an alveolar pattern. Similarly occasional fine fibrils will be seen between individual cells even in the most typical carcinoma. Errors of interpretation can be avoided by having adequate material available, and by a careful examination under high and low powers.

IV. Irradiation change

Irradiation, by causing an inflammatory reaction, necrosis and subsequent fibrosis, can mask the true reticulin pattern in a tumour particularly in biopsy specimens.

It is undoubtedly true that increasing anaplasia tends to render the patterns less well defined, but the writer is in agreement with Tudhope that the investigation is still worth doing in these cases. Particularly in anaplastic tumours it may be necessary to assess a predominating reticulin pattern and the observer should not be unduly influenced by occasional fibrillary areas in a carcinoma or alveolar areas in a sarcoma. Here again the importance of possessing adequate material cannot be overstressed. Even under the most favourable circumstances the method should be regarded as a link in a chain of evidence and not as an isolated test capable of proving the correctness of a diagnosis.

SUMMARY

1. The reticulin patterns of 164 carcinomas and 136 sarcomas have been examined.

2. The method is considered of limited but definite value in distinguishing primary and secondary carcinomas from sarcomas.

3. The importance of possessing adequate material for study is stressed.

I wish to thank Sir Stanford Cade for permission to study many cases under his care. I am indebted to Dr. A. D. Morgan for his advice, to the Librarian of St. Andrew's University for the loan of the Thesis, and to the Department of Medical Photography, Westminster Hospital.

REFERENCES

Ascher, F.—(1934) *Beitr. path. Anat.*, **92**, 1.
Bennett, G. A.—(1947) *J. Bone Jt. Surg.*, **29**, 259.
Brewer, D. B.—(1957) *J. Path. Bact.*, **74**, 371.
Cohn, M.—(1926) *Virchows Arch.*, **259**, 30.
Edelman, H.—(1925) *Ibid.*, **258**, 317.
Foot, N. C. and Day, H. A.—(1925) *Amer. J. Path.*, **1**, 431.
Gouyou, C.—(1946) *J. Urol. méd. chir.*, **53**, 473.
Haagensen, C. D. and Stout, A. P.—(1944) *Ann. Surg.*, **120**, 826.
Hsu, Y. K.—(1940) *Arch. Neurol. Psychiat., Chicago*, **43**, 901.
Jackson, D. S. and Williams, G.—(1956) *Nature, Lond.*, **178**, 915.
Kölliker.—(1852) Quoted by Tudhope.
Lumb, G. D. and Mackenzie, D. H.—(1956) *Brit. J. Surg.*, **43**, 380.
Niosi, F.—(1915) *Tumori*, **4**, 301.
Speciale, F.—(1924) *Ibid.*, **10**, 37.
Tillotson, J. F., McDonald, J. R. and Janes, J. M.—(1951) *J. Bone Jt. Surg.*, **33A**, 459.
Traut, H. F., Kuden, A. and Cadden, J. F.—(1939) *Amer. J. Obstet. Gynec.*, **38**, 798.
Tudhope, G. R.—(1937) M.D. Thesis University of St. Andrews.
Tuthill, C. R. and Meredith, J. M.—(1943) *Sth. med. J., Nashville*, **36**, 471.
Valls, J., Muscolo, D. and Schajowicz, F.—(1952) *J. Bone Jt. Surg.*, **34B**, 588.
White, W. C.—(1900) *Johns Hopk. Hosp. Bull.*, **11**, 209.
Willis, R. A.—(1953) ' Pathology of Tumours '. London (Butterworth & Co.).
Windrum, G. M., Kent, P. W. and Eastoe, J. E.—(1955) *Brit. J. exp. Path.*, **36**, 49.
Wright, C. J. E.—(1952) *J. Path. Bact.*, **64**, 585.

SPONTANEOUS AND TRANSPLANTABLE FUNCTIONAL TUMOUR OF THE ADRENAL CORTEX IN THE A × C RAT

RIGOBERTO IGLESIAS AND ELVIRA MARDONES

Instituto de Medicina Experimental del Servicio Nacional de Salud, Av. Irarrázaval 849, Santiago, Chile

Received for publication December 18, 1957

SPONTANEOUS tumours of the suprarenal cortex are rare in rodents. Adenomata were found in 3 out of 31,868 rats of various strains; the tumours appeared at 16 months or later; no transplantation was tried and no data about the functional condition of the 3 tumours are available (Curtis, Bullock and Dunning, 1931). Cortical adenomata in rats have been described also by Ratcliffe (1940), Heiman (1944) and Guérin (1954). Four animals with carcinoma of the cortex have been observed among 42 female rats of the Osborne-Mendel strain of rats fed a carcinogenic agent; this carcinoma was functional and transplantable (Mulay and Eyestone, 1955). A carcinoma was found in an aged non-castrated female mouse; the tumour was non-functional and there were no metastases (Dalton, Edwards and Andervont, 1943). Both adenomata and malignant tumours of the cortex were discovered in mice of the NH strain aged more than one year; in only one case with adenoma there was estrogenic action (Kirschbaum, Frantz and Williams, 1946; Frantz, Kirschbaum and Casas, 1947).

On the other hand tumours of the suprarenal cortex have been elicited by castration in the dog (Feodosiev, 1906), guinea-pig (Spiegel, 1939), mice (Fekete, Woolley and Little, 1941; Woolley, 1950, 1954; Gardner, 1941; Smith, 1945), rats (Houssay *et al.*, 1951–54) and hamsters (Keyes, 1949; Woolley, 1953). The work of the Woolley group in mice and of the Houssay group in rats deserves special mention.

In the present paper a spontaneous metastasizing and transplantable tumour of the suprarenal cortex which was found in a non-castrated female rat of the A × C strain will be described. The tumour, which was studied in four transplant generations, produced corticoids but no gonadal steroids. As far as we know no similar growth has been described in rats.

The Primary Tumour

The animals used in this study belonged to the A × C strain secured in December 1949 from the laboratory of Dr. Albert Segaloff, Alton Ochsner Medical Foundation, Tulane University, New Orleans. The rats were maintained on a diet of skimmed milk and whole grains (wheat and corn).

The primary tumour was found in July 1953 in a breeding female aged 550 days (Iglesias, 1954). There were no more litters in the first half of 1953; the animal attracted attention by pronounced loss of hair and emaciation; body weight at death was 114 g.

Necropsy was made about 12 hours after death. The left suprarenal was replaced by a tumour of 20 × 16 × 15 mm. (Fig. 1), of irregular surface but of

a colour similar to that of the normal suprarenal, with some grey zones. On section the tumour showed the same colour as the surface. Vascularization was abundant; there were no large necrotic zones. The consistency of the tumour was seemingly less than that of the normal suprarenal. The tumour did not adhere to any abdominal organ. The right suprarenal weighed only 9 mg., i.e. half of a normal right suprarenal in animals of the same age (in Table 8, Iglesias and Mardones, 1956). Nodules similar to the tumours were found in the spleen (Fig. 1) and liver the right lobe of which was almost totally replaced by tumoural masses.

Histology.—The tumour contained what seemed to be exclusively elements of the suprarenal cortex. There were two types of cells: large and clear cells arranged in nodules near the surface (Fig. 2a) or in the interior (Fig. 2b, 3); and small cells with scanty protoplasm and more dense nuclei. The tumoural nodules apparently compress the glomerulosa. The two types of cells probably originated from the fasciculata. Small necrotic zones were present at some places. The medulla was of an irregular shape seemingly due to compression by tumoural masses.

The nodules in the spleen, liver and also the lung consisted of cells intermediate between the large and small cells described above. The greater part of the liver was degenerate.

The tiny right suprarenal was atrophic. The three layers of the greatly reduced cortex could scarcely be recognized. The cells were exclusively of the small type. A nodule was present in the right suprarenal, probably a metastasis.

Results of Transplantation

A suspension of one part of tumour in 2 parts of saline was injected subcutaneously (dorsally, shoulder region) into 3 females, and intra-abdominally into 6 females, each animal receiving 0·2 c.c. There was no visible tumoural growth during more than 7 months. One animal died 219 days after intra-abdominal inoculation; a subcutaneous tumour continuing into the subjacent muscle was found at the site of inoculation. The whole abdominal cavity was full of tumours whose size varied between 2 and 20 mm. in diameter, besides many more tiny nodules (Fig. 4). In colour they were similar to the primary tumour. There was no liquid in the abdominal cavity. The weights of the suprarenals were 13 and 19 mg., i.e. somewhat less than in normal animals of the same age. The weight of the hypophysis was normal (8 mg.). The thymus was atrophic. The remaining 5 animals (Table I) which had been inoculated intra-abdominally died in the course of the next 24 days; tumours were found in all of them. In the course of the next 2 months tumours were found also in all the 3 animals with subcutaneous inoculation.

With the material taken several hours after death from the first animal, which died at 219 days, 16 animals were inoculated. Of these, 12 developed tumours; again with a latent period of about 8 months. The tumour took with fresh material invariably in both sexes in 100 per cent of the animals inoculated.

With fresh material from animals of the same or subsequent tumour generations the latent period dropped from the minimum of 8 months to a minimum of 2 months (Table I; compare E and F to A and C). A considerable decrease

TABLE I.—*One Hundred and Forty Intact Females and Males Inoculated with a Suspension of Tumour of Suprarenal Cortex*

	Generation of tumour	Group	Site of inoculation	Number of animals: Inoculated	With tumour	With metastases	Latent period* (days)	Survival (days)
			Donors died several hours before					
A	1	♀	Subcutaneous	3	3	1	235–279	336–462
B	1	♀	Intra-abdominal	6	6	2	—	219–243
C	2	♀	Subcutaneous	8	7	3	233–400	314–608
D	2	♀	Intra-abdominal	8	8	0	—	334–433
			Fresh material					
E	2, 3, 4	♀	Subcutaneous	53	53	18 (34%)	51–152	88–271
F	2, 3, 4	♂	,,	29	29	19 (65%)	58–259	161–395
G	2, 3, 4	♀	Intra-abdominal	18	18	1 (5%)	—	80–425
H	2, 3, 4	♂	,,	5	5	0	—	147–216
I	2, 3, 4	♀	Intramuscular	10	10	1 (10%)	81–225	129–299

* Determined by means of palpation.

in the latent period in the course of subsequent generations of a transplantable cortical tumour has previously been observed in mice (Dalton, Edwards and Andervont, 1943). In our work the latent period, with fresh material of the second generation, was much shorter than with material from a tumour of the same generation but taken several hours after death. One may argue that with the latter the latent period was prolonged due to a smaller number of surviving tumour cells being present in the inoculated material. With intramuscular inoculation the latent period was somewhat longer than with subcutaneous inoculation (Table I; compare E and I).

The transplanted tumour may reach a considerable size with a weight of 90 g. Histologically the tumour was throughout all the subsequent 4 generations very similar to the primary tumour though the size of the cells was seemingly greater than in the primary growth. There was no difference between the tumours growing in the abdominal cavity or subcutaneously.

There were, however, remarkable differences between the 2 sexes (Table II).

TABLE II.—*Comparative Latent Period and Survival in 82 Females and Males with a Subcutaneously Implanted Functional Suprarenal Tumour (Groups E and F of Table I)*

	Sex	51–100 (days)	101–200 (days)	201–270 (days)	270–400 (days)
Lat. period	♀	48 (91%)	5 (9%)	0	0
	♂	15 (52%)	12 (41%)	2 (7%)	0
Survival	♀	1 (2%)	33 (62%)	19 (36%)	0
	♂	0	6 (20%)	15 (52%)	8 (28%)

The latent period was much shorter in females than in males: it was of 51 to 100 days in the majority of females with subcutaneous inoculation and only in half of the males. More than 64 per cent of the females survived no more than 200 days; only 20 per cent of the males died at this period. As many as 28 per cent of the males were still alive when all the inoculated females were dead.

A difference in behaviour according to sex has been found also in mice, the tumour growing more rapidly in males than in females (Dalton, Edwards and Andervont, 1943)—just contrary to our tumour in the rat.

Metastases

Metastases occurred with subcutaneous inoculation in more than a third of the females and in more than half of the males (Table V). Indeed, frequency of metastases was not always so high (Iglesias and Mardones, 1958). A third of the metastases were in the lung, another third in the liver, and in the remaining third metastases were simultaneously present in lung (Fig. 7, 5) and liver (Fig. 8) or in lung or liver and spleen. It is remarkable that metastases were never found in the kidney, whereas with the ovarian tumour of the same strain metastases were present only in lung and kidney (Iglesias and Mardones, 1956). In general the number of metastatic nodules is enormous, their size varying from 1 to 20 mm. in diameter. The liver (Fig. 8) and especially the lung (Fig. 7) may be almost totally replaced by these metastatic nodules, and one is rather astonished to see the animals surviving so long. Metastases appear late, as with the transplantable ovarian tumour of the same strain (Iglesias and Mardones, 1956).

In experiments with subcutaneous inoculation metastases were found frequently also in the suprarenal cortex (Fig. 10). The individual metastatic nodules in the suprarenal are in general very small; but thanks to their number the size of the suprarenal may be greatly increased. The greatest size of a nodule in the suprarenal cortex was of about 3·5 mm. The size of the suprarenal with metastases varies greatly; we found a suprarenal of 4 mg. only containing 3 nodules, and another of 61 mg. containing 5 metastatic nodules (Fig. 10).

The comparative occurrence of metastases with the transplantable suprarenal tumour and the ovarian tumour of the same strain is of interest. Frequency varied with the site of inoculation: with the suprarenal tumour the frequency of metastases after subcutaneous inoculation was 7 to 10 times that after intra-abdominal or intramuscular inoculation; with the ovarian tumour the frequency was considerably greater with intramuscular than with subcutaneous inoculation (Table 5 in Iglesias and Mardones, 1956). Thus notwithstanding the existing differences, it is a fact that both with the transplantable ovarian and suprarenal tumours the presence or absence of metastases is not determined solely by the genetical condition of the tumour cell but also by the immediate environment in which the tumour cell is located.

The Functional Condition of the Suprarenal Tumour

Evidence as to the functional condition of our suprarenal tumour can best be summarized in the following scheme:

I. The suprarenal tumour produces corticoids:
   1. Progressive atrophy of the adrenals (Table III).
   2. Survival of adrenalectomized animals (Table IV).
   3. Toxic actions probably due to an excess of cortisone (loss of hair) and mineralocorticoids (oedema).
   4. Toxic actions probably due to an interference with the gonadotrophic activity of the hypophysis: atrophy of the gonads (Table V).

II. The suprarenal tumour does not produce oestrogens and androgens :

1. The uterus remains undeveloped in castrated females.
2. The seminal vesicles remain undeveloped in castrated males.

We have already mentioned atrophy of the suprarenals in animals with the suprarenal tumour. The longer the animals survive, the smaller are the adrenals (Table III ; Fig. 9), unless they increase due to metastases. In animals surviving only 88 days the weight of both adrenals may be as much as 42 mg., and the total weight of the adrenals may decrease to as little as 5 or 9 mg. in animals surviving 199 to 262 days.

TABLE III.—*Weight of Suprarenals in Animals with Subcutaneously Inoculated Functional Suprarenal Tumour*

Number of animals	Survival (days)	Weight of 2 suprarenals (mg.)
1	88	42
21	167 (112–251)	32–16
27	199 (137–270)	15–10
9	238 (199–262)	9–5

Notwithstanding all the variations as evidenced by Table III, the results are impressive. The only explanations one can offer here are : that the suprarenal tumour produces corticoids which control the hypophysis in such a way that delivery of corticotrophin is diminished ; or that the suprarenal tumour attracts all the corticotrophin available. The first is the more probable.

EXPLANATION OF PLATES

FIG. 1.—The primary suprarenal tumour in female, died at the age of 550 days. The tumour is on the left suprarenal (a). Beneath the tumour the spleen with large metastases (b). Right lobule of the liver almost wholly occupied by metastases (c)

FIG. 2.—Same animal as Fig. 1. Original suprarenal tumour. a. Near the surface of the tumour. Two types of cells : nodule of large and clear cells, surrounded by small cells with very poor protoplasm and very dense though not pycnotic nuclei. × 105. b. More to the interior of the tumour large nodule of large clear cells surrounded by small cells. The small cells apparently predominate numerically. × 105.

FIG. 3.—Same animal as Fig. 1 and 2. Group of large cells. Majority of cells with spheric nuclei and nucleoli. Minority of cells with dense nuclei. Note also disposition of cells in cords though this is not always the case (see Fig. 2). × 415.

FIG. 4.—Two hundred and nineteen days after intra-abdominal inoculation into intact female. 1st generation. Tumoural nodules on the epiplon. × 105.

FIG. 5.—Same animal as Fig. 7. Metastases in the lung. × 112.

FIG. 6.—Spleen with numerous metastases. Two hundred and fifty-five days after subcutaneous inoculation into intact female 3rd generation. There were metastases also in the liver and lungs.

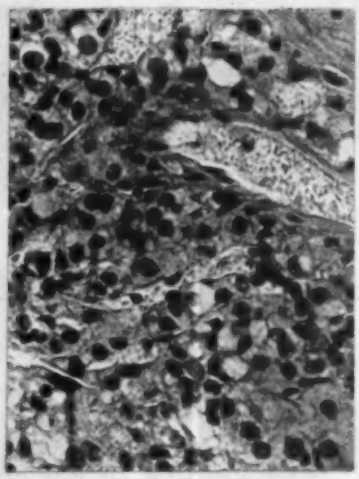
FIG. 7.—Numerous metastases in the lung. One hundred and thirty-five days after subcutaneous inoculation into castrated female receiving oestradiol. 3rd generation.

FIG. 8.—Liver with numerous metastases. Two hundred and sixty-seven days after subcutaneous inoculation into intact male. 3rd generation. The whole liver so to say transformed into lobulated tumoural mass.

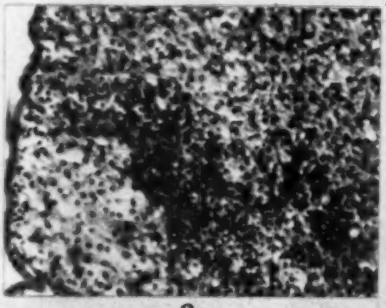
FIG. 9.—a. Suprarenal of normal female, aged 236 days. Weight of gland : 18 mg. b. Suprarenal of female 251 days after subcutaneous inoculation of suprarenal tumour. Weight of the gland : 8 mg. × 20.

FIG. 10.—Metastases in the suprarenal. Four hundred and sixty-two days after subcutaneous inoculation into intact female. Weight of suprarenal 61 mg. (1). × 14.

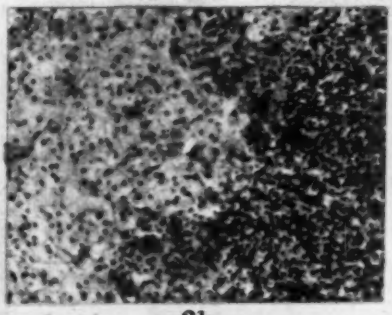
1



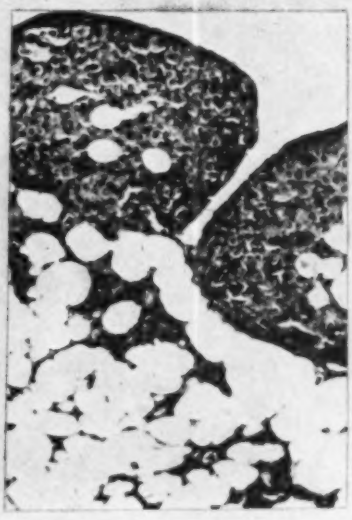
3



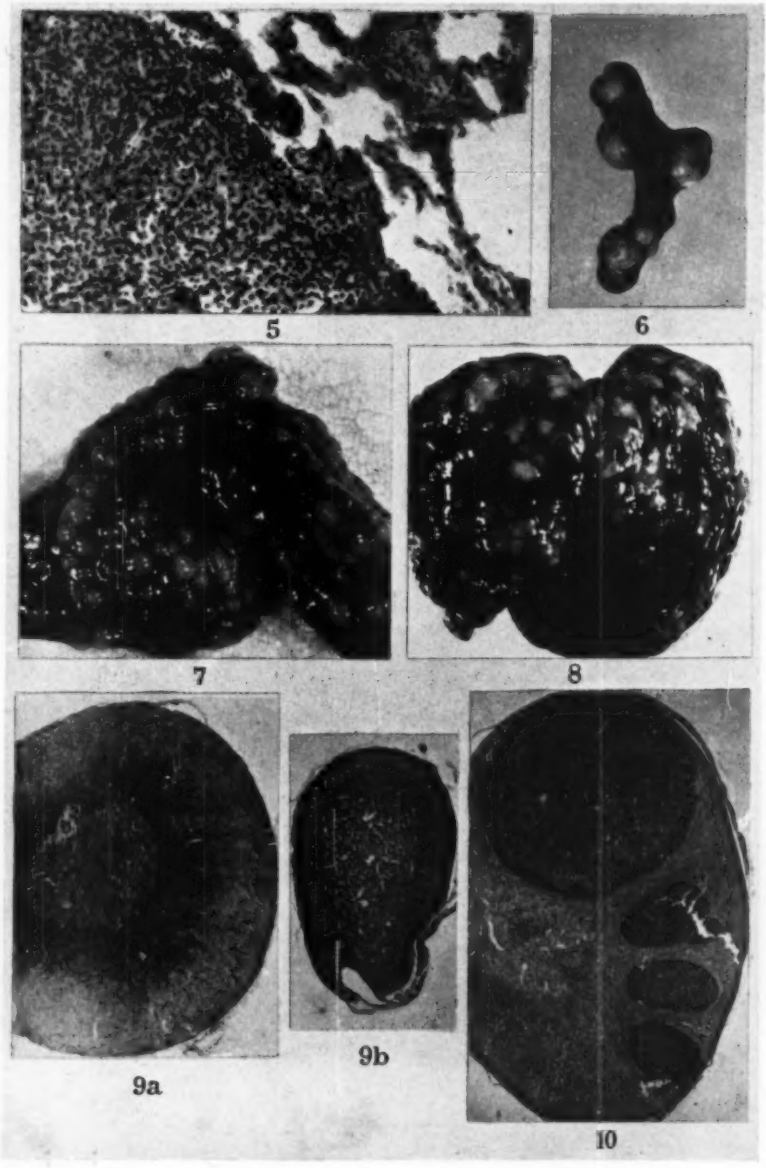
2a



2b



4



5

6

7

8

9a

9b

10

Even more convincing as to production of corticoids is the fact that animals with the suprarenal tumour survive adrenalectomy (Table IV). In none of the surviving animals were accessory suprarenals found.

TABLE IV.—*Seven Castrated Females Adrenalectomized* 108 *Days after Subcutaneous Inoculation of Suprarenal Tumour*

Number of animal	Survival after adrenalectomy (days)	"Volume" of tumour, at 100 days ($cm.^3$)	Weight of tumour, at necropsy (g.)	Metastases
1	2	8·0	9·2	0
2	44	1·3	7·4	0
3	77	9·4	38·0	Liver.
4	80	2·0	5·4	Liver, lung.
5	88	3·3	38·7	Liver, lung.
6	131	0·4	26·8	Liver, lung.
7	150	1·0	21·1	Liver, spleen, lung.

Another observation suggestive of the functional condition of the tumour or its metastases may be mentioned here. The cells of the tiny metastases found in the suprarenal are much larger than the cells of the cortex. The latter have been seemingly put out from their normal job by the cells of the tumour. No complete histochemical study of the suprarenals was made. But there was no sudanophilia and no birefringent material in the zona glomerulosa. Thus the suprarenals themselves apparently did not produce desoxycorticosterone (Greep and Deane, 1947) this corticoid being supplied by the tumour.

Corticoids were produced by the tumour seemingly in excess; most of our animals with suprarenal tumours showed loss of hair probably due to an excess of cortisone. In various animals the abdominal wall and the extremities were oedematous possibly due to an excess of mineralocorticoids.

There was also an atrophy of the sex glands, both in males and females (Table V). It seems reasonable to suppose that with an excess of corticoids the output of hypophyseal gonadotrophins is upset in the same way as is the output of corticotrophin.

TABLE V.—*Weight of Gonads in Normal Animals and in Animals with Functional Suprarenal Tumour*

Group	Number of animals	Age (days)	Weight of gonads: Right (mg.)	Weight of gonads: Left (mg.)	Weight of rt. seminal vesicle (mg.)
Normal males	5	302–438	963±167	1127±88	365±48
Males with tumour	6	303–346	148±5·2	156±7·2	33±3·6
Normal females	6	86–428	30±1·6	32±2·9	—
Females with tumour	14	160–360	12±1·1	13±0·9	—

Our spontaneous transplantable suprarenal tumour differs apparently in one fundamental aspect from the suprarenal tumours induced by castration. The experimental tumour produces oestrogen and androgen, as known since the classic work of the Woolley-Little group (Woolley and Little, 1946; Woolley, 1953). On the contrary, our spontaneous suprarenal tumour was not capable of maintaining the normal weight of the uterus or the seminal vesicles as shown

by the comparative condition of the uterus and of the seminal vesicles in intact and castrated animals with suprarenal tumours (see last column of Table V of Iglesias and Mardones, 1958). Though there might be some production of oestrogen or androgen by the tumour, the quantities produced were certainly not sufficient to maintain the weight of uteri and seminal vesicles at a normal level.

Our work with this tumour is still going on; at the moment the tumour is growing in the ninth transplant generation. We maintain several lines of the tumour. Some of these lines are probably not functional weight and aspect of the adrenals remaining normal notwithstanding the presence of the tumour.

SUMMARY

A tumour of the suprarenal cortex was found in an aged female rat of the A × C strain which had served for breeding. There were metastases in the lung, liver and spleen.

The tumour was transplantable, taking in 100 per cent of females or males, intact or castrated.

Frequency of metastases in the lung, liver, spleen, and the suprarenal was, with subcutaneous inoculation, 30 to 50 per cent; frequency was much smaller with intramuscular or intra-abdominal inoculation.

The primary tumour and also the tumours growing after transplantation produced corticoids as evidenced by the atrophy of the suprarenals and by the long survival of adrenalectomized bearers of the transplanted tumour.

The tumour does not produce oestrogen or androgen in quantities which would be sufficient to maintain the weight of the uterus and seminal vesicles at a normal level.

The authors wish to thank Miss Socorro Salinas for her enthusiastic technical help, and to Professor Alexander Lipschutz, Director of this Institute, for helpful criticism during the preparation of this paper.

REFERENCES

Curtis, M. R., Bullock, F. D. and Dunning, W. F.—(1931) *Amer. J. Cancer*, **15**, 67.

Dalton, A. J., Edwards, J. E. and Andervont, H. B.—(1943) *J. nat. Cancer Inst.*, **4**, 329.

Fekete, E., Woolley, G. and Little, C. C.—(1941) *J. exp. Med.*, **74**, 1.

Feodosiev, N. E.—(1906) *Russky Vrach*, No. 5, 135.

Frantz, M., Kirschbaum, A. and Casas, C.—(1947) *Proc. Soc. exp. Biol. N.Y.*, **66**, 645.

Gardner, W. U.—(1941) *Cancer Res.*, **1**, 632.

Greep, R. O. and Deane, H. W.—(1947) *Endocrinology*, **40**, 417.

Guérin, M.—(1954) 'Tumeurs Spontanées des Animaux de Laboratoire.' Paris (Legrand).

Heiman, J.—(1944) *Cancer Res.*, **4**, 32.

Houssay, A. B., Cardeza, A. F., Houssay, B. A. and Pinto, M. R.—(1954) *Rev. Soc. argent. Biol.*, **30**, 241.

Houssay, B. A., Cardeza, A. F., Pinto, R. M. and Burgos, M. H.—(1951) *Ibid.*, **27**, 56.

Idem, Cardeza, A. F., Houssay, A. B. and Pinto, R. M.—(1952) *Ibid.*, **27**, 315.

Idem, Houssay, A. B., Cardeza, A. F., Foglia, V. G. and Pinto, R. M.—(1953) *Acta Physiol. Lat.-Amer.*, **3**, 125.

IGLESIAS, R.—(1954) *Sixth int. Cancer Congr.*, Sao Paulo, p. 169.
Idem AND MARDONES, E.—(1956) *Cancer*, **9**, 740.—(1958) *Brit. J. Cancer*, **12**, 28.
KEYES, P. H.—(1949) *Endocrinology*, **44**, 274.
KIRSCHBAUM, A., FRANTZ, M. AND WILLIAMS, W. L.—(1946) *Cancer Res.*, **6**, 711.
MULAY, A. S. AND EYESTONE, W. H.—(1955) *J. nat. Cancer Inst.*, **16**, 723.
RATCLIFFE, H. L.—(1940) *Amer. J. Path.*, **16**, 237.
SMITH, F. W.—(1945) *Science*, **101**, 279.
SPIEGEL, A.—(1939) *Virchows Arch.*, **305**, 367.—(1939) *Klin. Wschr.*, **18**, 1068.
WOOLLEY, G.—(1950) *Rec. Progr. Horm. Res.*, **5**, 383.—(1953) *Anat. Rec.*, **115**, 381.—(1954) *J. nat. Cancer Inst.*, **15**, 717.
Idem AND LITTLE, C. C.—(1946) *Cancer Res.*, **6**, 712.

INFLUENCE OF HORMONES ON THE GROWTH OF A TRANSPLANTABLE SUPRARENAL TUMOUR

RIGOBERTO IGLESIAS AND ELVIRA MARDONES

Instituto de Medicina Experimental Servicio Nacional de Salud, Santiago de Chile

Received for publication December 18, 1957

THE evolution of the transplanted suprarenal tumour of A × C rats is different in the two sexes; the tumour grows more rapidly in intact females than in intact males, and females survive a shorter time than males (Table II in Iglesias and Mardones, 1958). The problem of the dependence of tumour growth on the gonads is of considerable interest. A similar dependence has been found also with other tumours: the transplantable ovarian tumour in mice (Mühlbock, 1952) and the transplantable ovarian tumour in our A × C strain of rats (Iglesias and Mardones, 1956*a*, 1956*b*). In the present paper results of a more detailed study of the dependence of the transplantable suprarenal tumour of A × C rats on the gonads will be given. The influence of gonadal steroids, of cortisone and of ACTH also has been studied. All experiments were made with the subcutaneous transplantation of a suprarenal tumour of the second transplant generation. The latent period was determined by palpation. The "volume" of the tumour is the product of three diameters measured with the caliper.

Results are summarized in Table I.

TABLE I.—*Twenty-four Animals with Subcutaneous Inoculation of Suprarenal Tumour Receiving Gonadal Steroids or Cortisone, Compared with 48 Intact and Castrated Animals with Tumours but Not Receiving Steroids. Seven Animals Receiving ACTH.*

Groups*	Steroid (μg./day)	Latent period (days)	Survival (days)	"Volume" tumour at 86 days (cm.³)	Weight of tumour at necropsy (g.)	Number of animals with metast.	Weight of the 2 suprarenals (mg.)	Weight of uterus and seminal vesicle (mg.)
1. Fem., intact	—	58	156	8·3	29	1	13·8	163
2. Fem., int. castr.†	—	52	175	7·8	34	2	12·5	142
3. Fem., castr.	—	67	216	1·0	35	0	13·3	80
4. Fem., castr.	Oestrad. (52)	85	147	0·7	16	3	26·0	416
5. Fem., castr.	Progest. (151)	67	221	0·44	34	2	12·7	80
6. Fem., castr.	Cortis. acet. (644).	52	162	1·7	18	1	11·6	108
								Sem. ves.‡ Right–left
7. Male, intact	—	64	235	0·6	41	3	10·6	33–29§
8 Male, int. castr.†	—	68	228	0·4	29	2	11·7	40–32
9. Male, castr.	—	66	223	0·5	38	2	11·6	15–13
								Uterus
10. Fem., castr.	ACTH	177	384	±	25	4	13·0	66

* Eight animals in each group, with the exception of group 10 with 7 animals only.
† Castrated at 86 days after inoculation.
‡ Right seminal vesicle with content; left seminal vesicle without content.
§ Weight of testicle: 152 mg.

Influence of the gonads

As seen from Table I the difference as to growth in intact females and males is very considerable. At say 86 days the volume of the tumour in intact females is many times greater than in intact males (compare groups 1 and 7). When comparing the evolution of the tumour in intact and castrated females it becomes fully evident that growth of the suprarenal tumour is greatly enhanced by the ovary (compare 1 and 3). On the contrary, there is no such influence of the male gonad (compare 7 and 9).

It is of great interest to compare these results with those obtained with the transplantable ovarian tumour in the same strain. Growth of the ovarian tumour is enhanced by the presence both of the ovary and the testicle (Iglesias and Mardones, 1956*a*) ; on the contrary, only the ovary is capable of enhancing the growth of the suprarenal tumour. But there is still another difference which is apparently fundamental : the differential response of the ovarian and suprarenal tumour of the same strain to gonadal steroids.

Influence of gonadal steroids

Steroids were absorbed from pellets implanted subcutaneously one day after inoculation. Absorption per day was determined from the loss of weight of the pellet. As seen from Table I, group 4, an average of 53 μg./day of oestradiol was absorbed. With quantities of oestradiol so large as 53 μg./day which were sufficient to cause uterine growth and suprarenal hyperplasia, the latent period was even longer than in castrated females without oestrogen (compare 3 and 4) ; at 86 days the tumour was still the same size as in castrated females not receiving oestrogen. Indeed survival was considerably shortened when oestradiol was given. At the first glance one might argue that oestradiol had some share in reconditioning malignancy of the suprarenal tumour, as is the case with the ovarian tumour (Iglesias and Mardones, 1956*b*). However, the shorter survival was most probably due to a pituitary tumour present in these females induced by the administered oestradiol. That death was not due to the suprarenal tumour is shown also by the following experiment : 8 castrated males not inoculated with the suprarenal tumour but receiving the same quantity of oestrogen survived only 162 (159–183) days, i.e. not more than the 8 inoculated castrated females receiving oestrogen (group 4) ; a large pituitary tumour similar to that of the females was found in all the 8 males. Thus, it seems reasonable to conclude that the growth of our suprarenal tumour is not enhanced by oestrogen and that malignancy of the suprarenal tumour is not reconditioned, contrary to what has been found with the transplantable ovarian tumour in the same strain. The same negative result was obtained in the present experiments with progesterone (compare groups 3 and 5). Growth of the transplantable ovarian tumour of the same strain was greatly stimulated by progesterone (Table I, group 7, in Iglesias and Mardones, 1956*b*). The objection could be made that in the experiments with the ovarian tumour much larger quantities of progesterone were given ; but the amount absorbed in the present work was still very considerable (group 5 in Table I).

Differential behaviour of the transplantable ovarian and suprarenal tumour of the same strain

Growth of both tumours depends on the ovary. The differential behaviour can be summarized in the following statement : whereas the influence of the

ovary on the evolution of the ovarian tumour and on the reconditioning of malignancy is due to the ovarian steroids, or to a steroid derived from these, reconditioning of the malignant growth of the suprarenal tumour in castrated females which is not obtained by gonadal steroids, is seemingly due to some other factor linked with the ovary so far unsuspected and unknown to us.

Comparative behaviour of the transplantable spontaneous suprarenal tumour and suprarenal tumours induced by castration

Both oestrogen and androgen are known to inhibit in mice the growth of suprarenal tumours induced by castration (Woolley, 1950). The final weight of our transplanted suprarenal tumour was smaller with oestrogen than with the presence of the ovary (Table I; compare groups 1 and 4). This difference cannot be explained by shorter survival as survival was not longer in intact females than in animals with oestrogen. The difference in the final weight of the tumour in groups 1 and 4 might be due to one of the three alternatives: (1) lack of the enhancing influence of the ovary, or (2) inhibition of growth as with oestrogen in animals with induced suprarenal tumours, or (3) shorter survival due to oestrogen.

Experiments with corticoids and A.C.T.H.—With cortisone (group 6) a certain amount of inhibition might have been produced, the final weight of the tumour being considerably smaller than in other groups with the same survival. But here again, as with oestradiol, the smaller final weight of the tumour might be due to lack of the enhancing influence of the gonad. Cortisone did not increase the frequency of metastases which occurs with other tumours (Agosin *et al.*, 1952). Desoxycorticosterone is known to inhibit the growth of induced suprarenal tumours in rats and mice (Houssay, Higgins and Bennett, 1951; Ambrad-Dominguez and Cardeza, 1953). Our own experience with this corticoid is not yet sufficient for us to venture any conclusion. Decidedly conflicting though at the same time stimulating results were obtained with the administration of corticotrophin. ACTH of Fredriksborg Chemical Laboratories was used and 4 I.U. were injected every second day; a total of 27 injections were given. An astonishing prolongation of the latent period and of survival took place. The animals survived 10 to 18½ months with an average of 13 months. We are unable to explain these results. It would be worth while to extend the experimental study of the action of corticotrophin on the suprarenal tumour.

SUMMARY

Growth of the transplantable spontaneous suprarenal tumour of A × C rats is slowed down and survival of the animals is prolonged by ovariectomy.

Growth of the tumour in intact males and also survival of the latter is similar to that in castrated females, and growth in males is not influenced by castration.

Metastases are more frequent in males than in females, due probably to longer survival of males.

The influence of the ovary on the growth of the tumour is apparently not due to oestrogen or progesterone.

With ACTH, growth of the tumour was inhibited: the latent period and survival were considerably prolonged.

It gives us pleasure to thank Miss Socorro Salinas for technical help, and to Professor Alexander Lipschutz, Director of the Institute, for valuable advice in writing the manuscript.

REFERENCES

AGOSIN, M., CHRISTEN, R., BADINEZ, O., GASIC, G., NEGHME, A., PIZARRO, O. AND JARPA, A.—(1952) *Proc. Soc. exp. Biol. N.Y.*, **80**, 128.
AMBRAD-DOMINGUEZ, N. AND CARDEZA, A. F.—(1953) *C.R. Soc. Biol. Paris*, **147**, 153.
HOUSSAY, A., HIGGINS, G. M. AND BENNETT, W. A.—(1951) *Cancer Res.*, **11**, 297.
IGLESIAS, R. AND MARDONES, E.—(1956*a*) *Cancer*, **9**, 740.—(1956*b*) *Cancer Res.*, **16**, 756.—(1958) *Brit. J. Cancer*, **12**, 20.
MÜHLBOCK, O.—(1952) *Ciba Found. Coll. Endocrinol.*, **1**, 112.
WOOLLEY, G. W.—(1950) *Cancer Res.*, **10**, 250.

THE ROLE OF THE OVARY IN THE INDUCTION OF TUMOURS BY THE LOCAL APPLICATION OF 9,10-DIMETHYL-1,2-BENZANTHRACENE TO THE GENITAL TRACT OF RATS

A. GLUCKSMANN AND CORA P. CHERRY*
From the Strangeways Research Laboratory, Cambridge

Received for publication November 12, 1957

THE incidence of cancer of the uterine cervix appears to be affected by hormonal factors and there is also some suggestion that the tumour type may be influenced by the endocrine state (Glucksmann and Cherry, 1956). Experimentally it has been possible to induce cancer of the uterine cervix by hormonal treatment as well as by hydrocarbons. The literature on this subject has been well reviewed by Gardner (1953) and von Haam and Scarpelli (1955). Most of the work has been done with mice in which with the exception of one strain (Gardner and Pan, 1948) the incidence of spontaneous cancers of the cervix is rare. The hydrocarbons proved more effective in tumour production than the oestrogens and the resulting tumours were mainly squamous-cell carcinomas. Sarcomas were rare and none are mentioned in von Haam's report of the literature nor by Reagan, Wentz and Machicao, (1955) and Murphy (1953). Gardner and Pan (1948) observed 1 sarcoma and 12 carcinomas in the PM strain of mice in which the tumour occurred " spontaneously ". In hybrids of that strain treated with oestrogen ± androgens, only carcinomas were found (Pan and Gardner, 1948*a*). On subcutaneous grafting of uterine cervices and cornua together with methylcholanthrene crystals, Pan and Gardner (1948*b*) obtained in strain A and hybrid mice almost as many sarcomas as carcinomas.

Rats have not been used extensively for these studies. McEuen obtained a few cancers of the uterine cervix after prolonged treatment with oestrogens. Pfeiffer (1949) reports 4 leiomyomas and an adenocarcinoma of the uterus in female rats in which testes had been grafted shortly after birth. Vellios and Griffin (1957) using a technique similar to that of Murphy (1953) and of Reagan *et al.* (1955) induced 13 tumours of the cervix in 39 rats in 28 weeks. One of these tumours was a carcinosarcoma, the others were carcinomas. Using spermicidal contraceptives applied per os or per vaginam daily for 14 to 18 months to rats kept on a low protein diet Hoch-Ligeti (1957) induced 21 tumours in 95 animals. These tumours are described as " fibrous or adenomatous polyps covered by multilayered squamous epithelium . . . Three tumours had characteristics of sarcomas, with invasion of the uterine wall, irregular mitoses, giant cells and marked anaplasia ". In addition (?) an angiofibrosarcoma and an epidermoid carcinoma are given as illustrations. The rats on the low protein diet showed liver damage which may have impaired the detoxication of oestrogens. It is not clear from the account how many of the tumours were sarcomas or carcinomas and what role, if any, interference with the oestrogen metabolism played in the induction of the uterine tumours.

* Working with a grant from the British Empire Cancer Campaign.

The experiments described in the present paper were undertaken to study the influence of endocrine factors on the incidence and type of tumours of the female genital tract; 9,10-dimethyl-1,2-benzanthracene was applied locally to the vagina and vulva of virgin rats, of castrated rats and of castrated rats treated with oestradiol.

MATERIALS AND METHODS

Female hooded rats were used throughout this study and their age at the beginning of the experiments was between 3 and 4 months. 9,10-Dimethyl-1, 2-benzanthracene (DMBA) obtained from Messrs. L. Light and Co. was the carcinogenic agent and a 1 per cent solution in acetone was used.

Twenty-eight virgin animals were painted intravaginally once weekly with DMBA for 28 weeks and thereafter the painting was done twice weekly for a further 27 weeks after which the experiment ended. Twelve virgin rats, which served as controls, were painted with acetone once weekly for 28 weeks and thereafter twice weekly.

Forty rats were castrated between the ages of two and three months and were used for the experiments one to two months later. Sixteen of these castrated animals were painted intravaginally twice weekly with a 1 per cent solution of DMBA in acetone. Another 16 rats were treated as above and in addition were given twice weekly intramuscular injections of 1μg. oestradiol monobenzoate (Organon) in olive oil, the injections being given the day before the painting with the carcinogen. Eight castrated rats served as controls and were painted intravaginally twice weekly with acetone.

The cervix and vagina were painted by means of a cotton wool swab on the end of a galvanised wire. The vagina was stretched open by dorsal flexion of the tail, the swab was inserted and the cervix and vagina painted by means of a rotary motion. By this method of painting the vulva was contaminated with DMBA.

Animals were killed from 200 to 410 days after the beginning of the experiment and the uterus, cervix, vagina and vulval skin were removed and in the virgin rats the ovaries were also taken out. Six of the virgin rats and 3 of the castrated rats in the experimental groups had to be killed for other conditions before 200 days and they have not been included in the experimental results.

The tissues were fixed in Zenker's fluid or Susa solution and embedded in paraffin. Sections were cut at 8 μ and stained with haematoxylin and eosin, by the periodic acid-Schiff technique with and without diastase digestion, with Southgate's mucicarmine stain or with van Gieson's stain.

A quantitative analysis was made by cell counts and by measurements of the vaginal and vulval epithelium and of the stroma of the vagina and uterus in the various experimental and control groups. Straight regions of the epithelium without carcinomas or warts were selected, the height of the epithelium was measured and total cell counts were made over a unit length of 200 μ. In the vagina the width of the stroma was taken from the lower edge of the epithelium to the inner muscle layer and in the uterus from the epithelium to the inner circular muscle layer.

RESULTS

(a) *Acetone painting.*—The repeated intravaginal applications of acetone caused some thickening of the vaginal epithelium and marked cornification in

both virgin and castrated rats. Keratohyaline granules were present in a well-developed stratum granulosum which was covered by fairly thick layers of keratin (Fig. 6). No effect of the acetone painting on the epithelium of the vulva could be detected (Fig. 1). Apart from occasional slight inflammatory infiltrations the vaginal stroma showed no abnormalities. None of the acetone-painted rats produced tumours in either the vagina or the skin at the introitus vaginae (vulva).

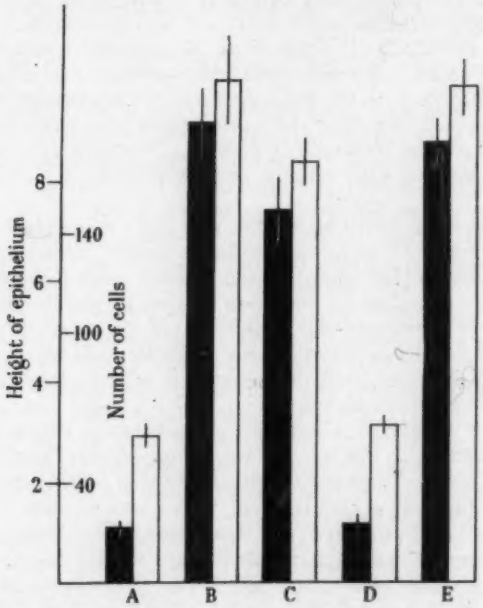


FIG. 1.—The average height ■ and the average number of cells □ per unit length of vulval epithelium. The standard deviations are indicated by lines in each column.

A.—Acetone treatment of castrated rats.
B.—DMBA treatment of castrated rats.
C.—DMBA + oestradiol treatment of castrated rats.
D.—Acetone treatment of virgin rats.
E.—DMBA treatment of virgin rats.

(b) *Castration.*—The castrated control rats painted with acetone showed an atrophy of the vaginal epithelium as compared with the acetone painted virgin rats. Measurements of the height of the epithelium (Fig. 2) indicate that the average thickness in the castrated animals was only 58 per cent of that in virgin rats. The total cell count per unit length of the epithelium was also reduced but only to 89 per cent. This disparity between the reduction in the height of the epithelium and in the total cell count can be attributed to a smaller average size of the epithelial cells in the castrates so that in a given volume of tissue more of the smaller cells were present than of the larger cells in the virgin rats.

The epithelium of the vulva in castrated and virgin rats painted with acetone showed the same thickness and total number of cells (Fig. 1) and is apparently not affected by castration.

The vaginal stroma of the castrated animals was thinner and less cellular than that of the virgin rats. Measurements show a relative reduction in width of the stroma by castration to 48 per cent (Fig. 3). An even greater reduction in width

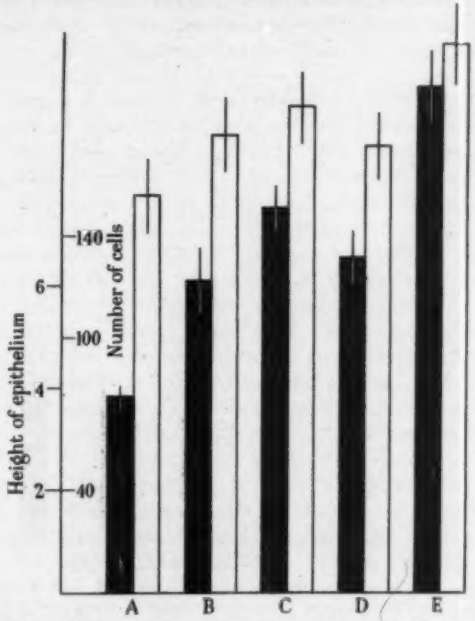


FIG. 2.—The average height ■ and the average number of cells □ per unit length of vaginal epithelium. The standard deviations are indicated by lines in each column.

A.—Acetone treatment of castrated rats.
B.—DMBA treatment of castrated rats.
C.—DMBA + oestradiol treatment of castrated rats.
D.—Acetone treatment of virgin rats.
E.—DMBA treatment of virgin rats.

of the uterine stroma was observed after castration (Fig. 3), the width of the stroma in the castrates being only 40 per cent of that in virgin rats (Fig. 7–8). The oestrogen treatment restored the width of the vaginal and uterine stroma to that of the virgin rats (Fig. 9).

Castration thus decreases the volume of uterine stroma, vaginal stroma and vaginal epithelium in that order, but does not affect the epithelium of the vulva (Fig. 1).

(c) *DMBA painting on virgin rats* was followed by marked thickening of both vaginal and vulval epithelium. The vaginal epithelium showed marked cornification and had some downward projections. In only two animals did this hyperplasia proceed to tumour formation : one rat had a papilloma (Fig. 10) and

another a squamous-cell carcinoma (Fig. 11) arising in the vaginal epithelium. Both these animals were killed 380 days after the beginning of treatment.

The hyperplasia of the vulval epithelium was much more striking than that of the vagina and was followed by the appearance of warts and of squamous-cell carcinomas in 95 per cent of the 22 animals at risk.

While the hyperplasia of the vagina caused an increase in the total number of cells to 124 per cent of that in the acetone-painted controls (Fig. 2) and an increase to 146 per cent in the height of the epithelium, the comparable figures for the

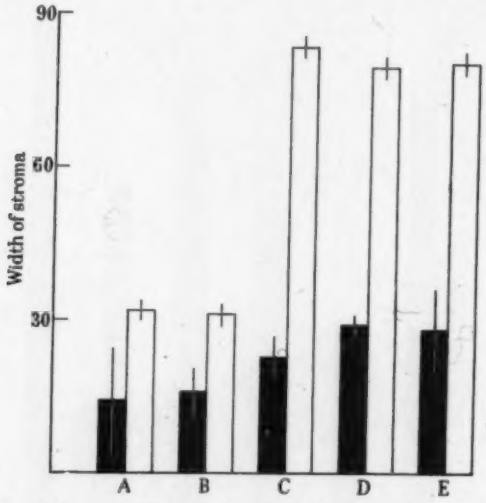


FIG. 3.—The average width of the stroma in the vagina ■ and in the uterus □. The standard deviations are indicated by lines in each column.

A.—Acetone treatment of castrated rats.
B.—DMBA treatment of castrated rats.
C.—DMBA + oestradiol treatment of castrated rats.
D.—Acetone treatment of virgin rats.
E.—DMBA treatment of virgin rats.

vulval hyperplasia are 325 per cent and 800 per cent respectively (Fig. 1). The far greater increase in width of the epithelium than in cell count is accounted for by changes in cell size.

The first tumour of the vulva was histologically identified as a squamous-cell carcinoma in a rat killed 253 days after the beginning of treatment. Of the 22 rats surviving for 250 or more days 21 had tumours at the vulva of which 15 were carcinomas and 6 were warts. The tumours were often multiple and large necessitating the killing of the animal. Apart from squamous-cell carcinomas some basal-cell carcinomas arising in the hair follicles were found. The papillomas tended to precede the appearance of carcinomas which often arose in them. Of the 6 warts, 4 were found in rats killed before the 350th day while of the 15 carcinomas only 7 were found before that time. The animals with warts were

killed, because of tumours in the vagina, probably before the malignant change occurred.

Apart from the carcinomas of the vulva the most striking change produced by DMBA painting was the appearance of sarcomas in the stroma of the vagina. Of the 22 rats at risk 16 were found to have sarcomas of the vaginal wall. The first tumour was found after 283 days i.e. 30 days later than the first carcinoma of the vulva, but the subsequent appearance of tumours was very similar to that of carcinomas of the vulva: 8 appeared before the 350th day and the average time for sarcomas on histological verification was 333 days as against 332 days for the carcinomas of the vulva.

The sarcomas varied in extent from growths involving the vagina, uterus, bladder and other pelvic structures to microscopic lesions extending for only a few sections. In type they were mostly cellular fibrosarcomas containing numerous multinucleate giant cells (Fig. 12, 13). The smallest lesions arose in perivascular infiltrations in the vaginal stroma in which the normal fibrous structure had disappeared, and the cellular infiltrate was characterised by the appearance of large cells with prominent nuclei containing coarse chromatin granules (Fig. 14, 15). These cells were found in varying proportions in the fully developed and invasive sarcomas (Fig. 13). Some of the smaller lesions seemed to be composed mainly of such giant cells as for instance the small lesion illustrated in Fig. 16. In others there was some fibre formation leading to the appearance of fibromatous regions in some of the tumours (Fig. 10). The vaginal and cervical epithelium over these lesions was usually hyperplastic and showed numerous projections (Fig. 12). With the growth of the sarcomas the epithelium was stretched and ulceration followed.

The early effects of DMBA-painting on the vaginal stroma were not striking. Apart from occasional slight inflammatory changes no generalised effect could be detected. Tumour formation was a late effect and focal in that it arose in some perivascular infiltrates. The thickness of the vaginal as of the uterine stroma was unaffected by the treatment (Fig. 3).

Treatment with DMBA thus causes the occurrence of carcinomas and warts in the vulva, of sarcomas in the vaginal stroma, some hyperplasia of the vaginal epithelium with the rare occurrence of a papilloma and of a carcinoma in this region. The uterine stroma is not affected by the painting. In some rats subjected to prolonged treatment with DMBA squamous metaplasia of endometrial glands and epithelium was observed.

(d) *DMBA-painting of castrated rats* was followed as in virgin rats by marked thickening of both the vulval and vaginal epithelium. While the hyperplasia in the vulva equalled that in virgin rats (Fig. 1), that of the vaginal epithelium was less pronounced than in virgin rats as shown by the total cell counts and the height of the epithelium (Fig. 2). In addition there were fewer downward projections of the epithelium in the treated castrates than in the treated virgin rats.

The differential effect of DMBA painting on the vaginal and the vulval epithelium is indicated by the following figures: the increase in total number of cells was only 115 per cent in the vagina as against 345 per cent in the vulva and the height of the vaginal epithelium was increased to 160 per cent as against 900 per cent in the vulva.

No epithelial tumours whether benign or malignant were found in the vagina during the experiment, while 11 of 13 rats at risk had tumours of the vulva.

All these tumours were carcinomas and were found in animals killed 344 to 406 days after the beginning of treatment. As Fig. 4 shows the vulval tumours tended to appear slightly later than in the virgin rats. Whether this difference is real remains open to doubt since the virgin rats were killed earlier because of the appearance of sarcomas in the vagina.

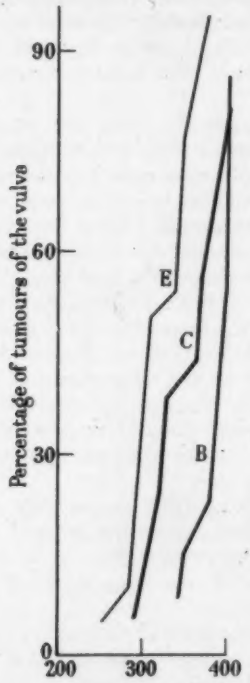


FIG. 4.

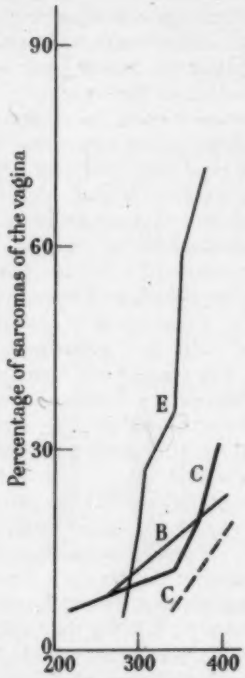


FIG. 5.

FIG. 4.—The percentage of tumours of the vulva after DMBA-painting in castrated rats (B), in castrated rats given oestradiol (C) and in virgin rats (E).

FIG. 5.—The incidence of sarcomas of the vagina after DMBA-painting in castrated rats (B), in castrated rats given oestradiol (C) and in virgin rats (E), and of pre-sarcomatous lesions (— — —) in castrated rats given oestradiol.

Only 3 of the 13 rats at risk developed sarcomas in the vaginal stroma, the first of which was found after 260 days. In another animal there was a tumour of the adrenal and the uterus and cervix showed signs of oestrogen stimulation. The tumours were fibrosarcomas similar to those observed in the virgin animals.

No significant effect of DMBA painting on the width of the vaginal or uterine stroma could be found (Fig. 3). The vaginal stroma tended to be rather thin, with few cells and somewhat hyalinised.

Treatment of castrated rats with DMBA is followed by the appearance of

carcinomas of the vulva, of a few sarcomas in the vaginal stroma and by hyperplasia of the vaginal epithelium.

(e) *DMBA painting of castrate rats given oestradiol* is followed by marked thickening of both the vulval and vaginal epithelium (Fig. 1, 2). This was more pronounced in the vulva than in the vagina, the increase in total number of cells for the vagina being 122 per cent and for the vulva 290 per cent. The increase in height of the epithelium was 198 per cent for the vagina and 670 per cent for the vulva. The smaller increase in total number of cells than in height of the epithelium is again accounted for by the increase in cell size. The vaginal hyperplasia in this group was almost as great as in the group of virgin rats, but no papillomas or carcinomas resulted in the vagina.

Of 16 rats at risk 13 had tumours at the vulva of which 9 were carcinomas and 4 warts. Three of the 4 warts and 3 of the 9 carcinomas were found in animals killed before the 350th day.

The width of the vaginal stroma and that of the uterine stroma equalled that of the virgin animals (Fig. 3) whether treated with acetone or with DMBA. Since in the latter no difference between acetone and DMBA-treated rats was found, the increase in width of the stroma of these castrated animals must be attributed to the oestradiol treatment rather than to the DMBA painting.

As in the virgin rats there was some perivascular infiltration in the vaginal stroma after DMBA application and from these lesions or associated with them sarcomas developed in 5 of the 16 rats at risk. The first of these tumours was found in a rat killed after 218 days. The other tumours developed more slowly than in the virgin rats treated with DMBA (Fig. 5).

In addition to these sarcomas, 3 rats had lesions in the vaginal stroma consisting of perivascular infiltrations of large cells resembling those found in early sarcomas and surrounded by some fibre-forming fibroblasts (Fig. 17–19). These lesions may represent a pre-sarcomatous state in that they have some of the characteristics of the early sarcomas but lack evidence of rapid proliferation and are less cellular than sarcomas. The incidence of these lesions which were not found in either the castrated or the virgin rats treated with DMBA is indicated by the broken line in Fig. 5.

Thus the combined treatment of castrates with oestradiol and DMBA is followed by the appearance of carcinomas of the vulva, of some sarcomas and some presarcomatous lesions in the vaginal stroma and by hyperplasia of the vaginal epithelium. The incidence of vaginal sarcomas and the extent of hyperplasia of the vaginal epithelium is intermediate between the DMBA-treated castrate and virgin rats.

DISCUSSION

In mice (Pan and Gardner, 1948*a*, 1948*b*; Gardner, 1953; Murphy, 1953; *Reagan et al.*, 1955; von Haam and Scarpelli, 1955) as well as in rats (McEuen, 1938; Pfeiffer, 1949; Vellios and Griffin, 1957) local application of carcinogens or treatment with oestrogens has led to the appearance of carcinomas of the vagina or cervix rather than of sarcomas. Only Pan and Gardner (1948*b*) observed in mice an almost equal incidence of carcinomas and sarcomas in uterine cervices which were grafted with methylcholanthrene crystals into litter mates. In their experiments the carcinomas appeared earlier than the sarcomas. They observed

that the carcinomas grew at a fairly uniform rate while the sarcomas tended to grow more abruptly and progressively. They do not comment on the site of origin of the sarcomas.

In our experiments the sarcomas of the vagina greatly exceeded the carcinomas even in virgin rats where the proportion of vaginal carcinomas to sarcomas was 1 : 16 or 2 : 16 if the vaginal papilloma is included. Furthermore the papilloma and the carcinoma of the vagina appeared later than the sarcomas. On the vulva, on the other hand, warts and carcinomas appeared early—the first warts being noticed by about 200 days—and no sarcomas were found there in these experiments.

The difference in tumour production by the vaginal and vulval epithelium (Table I) is correlated with the degree of hyperplasia induced by painting. While the total number of cells per unit length of the vagina rises to only 115–124 per cent that of the vulva increases to 290–345 per cent and the difference in the increase in the height of the epithelium is even greater : 146–198 per cent for the vagina and 670–900 per cent for the vulva. Furthermore in the vagina the hyperplasia is more marked in the virgin rats than in either the castrate or the castrated rats receiving oestradiol ; in the virgin rats only, are found a carcinoma and a papilloma though the other two groups were exposed to risk for a longer period. These findings suggest (1) a correlation between the induced hyperplasia and

EXPLANATION OF PLATES

FIG. 6.—The vagina of a virgin rat 10 months after acetone painting. The epithelium shows marked cornification and a well-developed stratum granulosum covered by fairly thick layers of keratin. (H. & E.) × 65.

FIG. 7.—A transverse section through a uterine horn of a virgin rat 13 months after acetone painting of the vagina. (H. & E.) × 26.

FIG. 8.—A transverse section through a uterine horn in a castrated rat 13 months after DMBA painting of the vagina. Note the marked reduction in width of the stroma as compared with Fig. 7. (H. & E.) × 26.

FIG. 9.—A transverse section through a uterine horn 9 months after DMBA painting of the vagina in a castrated rat given oestradiol. The width of the stroma is equal to that of the vigin rat in Fig. 7. (H. & E.). × 30.

FIG. 10.—The vagina of a virgin rat painted with DMBA for 13 months shows a papilloma and a sarcoma with marked fibre formation. (H. & E.) × 19.

FIG. 11.—A squamous-cell carcinoma in the vagina of a virgin rat painted with DMBA for 13 months. (H. & E.) × 32.

FIG. 12.—The vagina of a virgin rat painted with DMBA for 10 months shows downward projections of the hyperplastic epithelium and a large cellular fibrosarcoma with numerous multinucleate giant cells. (H. & E.) × 32.

FIG. 13.—An area in Fig. 12 at higher magnification illustrates giant cells with large, often multiple and hyperchromatic nuclei and some abnormal mitotic figures. (H. & E.) × 150.

FIG. 14.—An early sarcoma associated with a perivascular infiltration in the vagina of a virgin rat painted with DMBA for 10 months. (H. & E.) × 67.

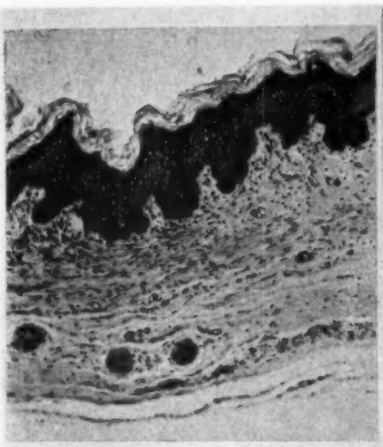
FIG. 15.—The perivascular infiltration in Fig. 14 at higher magnification with the characteristic large cells with hyperchromatic nuclei. (H. & E.) × 520.

FIG. 16.—The vagina of a virgin rat painted with DMBA for 12 months showing a small sarcomatous lesion composed mainly of giant cells with large prominent nuclei. (H. & E.) × 210.

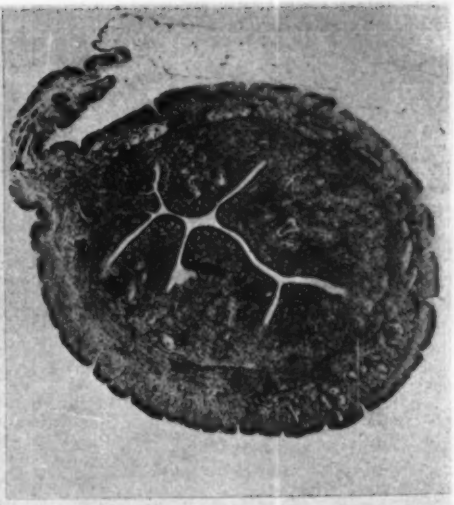
FIG. 17.—The vagina of a castrated rat given oestradiol and painted with DMBA for 12 months showing the extent of a " pre-sarcomatous " lesion associated with a perivascular infiltration. (H. & E.) × 33.

FIG. 18.—An area in Fig. 17 at higher magnification to show the perivascular infiltration and the " pre-sarcomatous'" lesion consisting of large cells with hyperchromatic nuclei and associated fibroblasts in thickened connective tissue. (H. & E.) × 90.

FIG. 19.—The large cells of the " pre-sarcomatous " lesion of Fig. 17 and 18 are shown at higher magnification. The large cells with prominent nuclei and coarse chromatin granules resemble those seen in the sarcomas (Fig. 13, 15 and 17). (H. & E.) × 245.

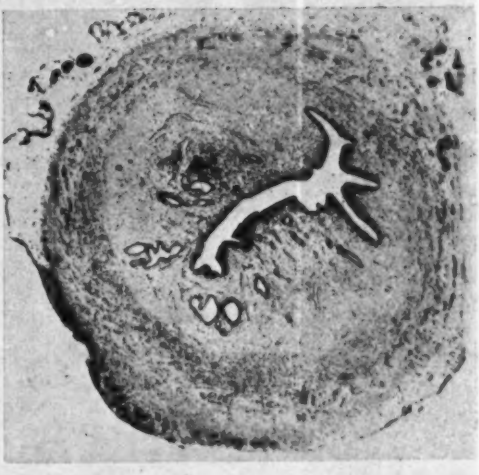


6



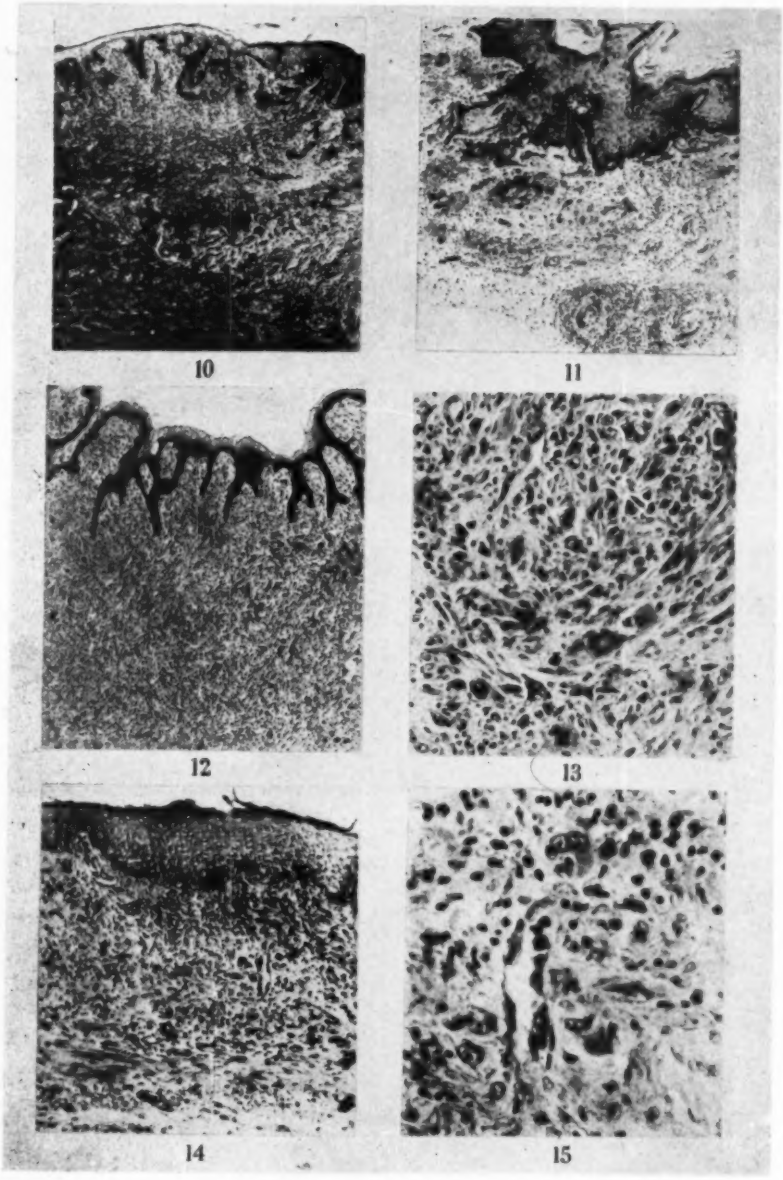
7

8



9

Glucksmann and Cherry.



10

11

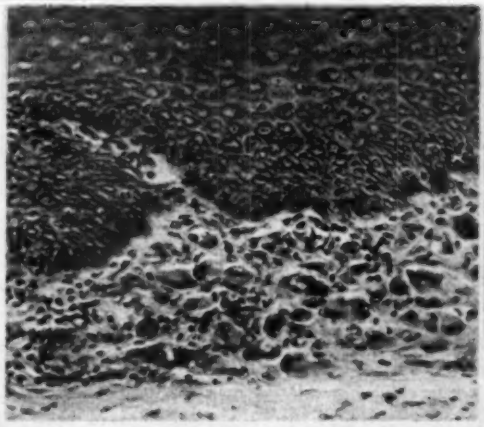
12

13

14

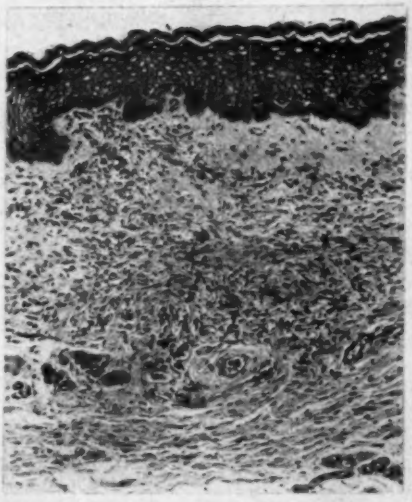
15

Glucksmann and Cherry.

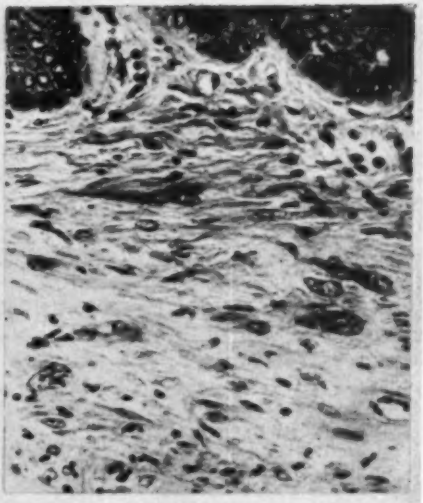


16

17



18



19

subsequent tumour formation and (2) an influence of ovarian activity on the hyperplasia of the vagina following DMBA painting.

TABLE 1

Experiment	Castration	Treatment with: Acetone	DMBA	Oestradiol	Number of rats: At risk	With tumours in: Vagina	Vulva
A	+	+	−	−	8	0	0
B	+	−	+	−	13	3	11
C	+	−	+	+	16	5 (+3)*	13
D	−	+	−	−	12	0	0
E	−	−	+	−	22	18†	21

* 3 pre-sarcomatous lesions.
† 16 sarcomas, 1 carcinoma and 1 papilloma.

The difference in the reaction of the vaginal and vulval epithelium may be due to dosage of DMBA, to the existence of a mucous barrier in the vagina, to a greater toxic effect of the painting on the vagina or to an inherent difference in the reactivity of the vaginal and vulval epithelium. Of these various possibilities the mucous barrier might be excluded in view of the fact that sarcomas appeared in the vaginal stroma and that therefore the hydrocarbon has probably penetrated the epithelium in quantities sufficient for tumour induction. We have found little evidence of ulceration of the vagina during the experiment and are thus inclined to attach little weight to the toxic effect of the hydrocarbon on the vaginal epithelium : i.e. that the epithelium was destroyed by the hydrocarbon which passing through to the naked stroma caused sarcomas in this localisation. Since a carcinoma and a papilloma have been induced in the vagina, the epithelium is not entirely refractory to the hydrocarbon and the mucous barrier is not sufficient to protect the vagina. The actual difference in dosage in the vaginal and vulval epithelium is difficult to assess owing to the differences in surface properties of the haired skin of the vulva and the cornified surface of the vagina. We incline to the view that the difference in carcinoma induction in the vagina and vulva is due to differences in the reactivity of the two tissues. The presence of hair follicles in the vulva may contribute to the greater sensitivity of the vulva since a number of the tumours arise in the follicles (Wolbach, 1951).

The induction of sarcomas in the vagina is obviously influenced by the presence and activity of the ovary (Table I). The incidence of sarcomas in castrated rats is significantly lower than in virgin animals painted with DMBA, and the tumours appear later. The presence of an adrenal tumour in one of the castrated rats with a vaginal sarcoma suggests that adrenal corticoids may play some role in the induction of vaginal sarcomas in the castrate or that in this animal at least there was oestrogenic stimulation. The incidence of vaginal sarcomas is increased by oestradiol treatment but not to the level in the virgin rats. The oestradiol was applied twice weekly in order to have variations in the oestrogen levels comparable to the normal oestrus cycle. The effect of this treatment on the width of the uterine and the vaginal stroma shows that the dose level was similar to that of virgin rats though it was probably not quite sufficient for the stimulation of the vaginal epithelium.

The results suggest that oestradiol is not the, or not the only, ovarian hormone which influences the sarcoma production in the vagina as shown by the difference between the castrated and virgin rats. The oestradiol treatment produced some presarcomatous lesions in the vaginal stroma on painting with DMBA in the castrated rats, but whether these lesions are reversible or whether they progress to invasiveness, remains to be investigated. The fact that they occur only in this group (Table I), suggests that oestradiol treatment helps to initiate the carcinogenic process in combination with the DMBA-painting, but that the " promoting " action which occurs in the virgin rats and probably quickly converts the presarcomatous lesion into a sarcomatous one is missing. The observation by Pan and Gardner (1948*b*) that the sarcomas start to grow suddenly and progressively, may be taken as indication why the presarcomatous lesions are not observed in the virgin rats after DMBA painting.

The median latent period for sarcoma production in virgin rats was 11 months, and 12 months in the castrated rats treated with oestrogen. DMBA-painting of the interscapular skin of normal rats induced carcinomas after a median latent period of 9 months and sarcomas after 11 months (Boag and Glucksmann, 1956). The incidence of carcinomas of the skin was also more numerous than that of sarcomas: 73 per cent of all tumours being carcinomas and only 27 per cent sarcomas. In the present experiment sarcoma induction in the vaginal stroma of virgin rats is of the same order of duration as for the skin, but the carcinoma induction is almost missing. In the vulva, on the other hand, carcinomas only and no sarcomas were induced. Whether the differences in types of tumours arising after DMBA-painting at different sites is due to difference in effective dosage, i.e. to differential dilution and absorption of the carcinogen, or to inherent differences in the responsiveness of the tissue at various sites, remains to be decided.

SUMMARY

1. The effect of local applications of DMBA on the female genital tract of virgin rats, of castrated rats and of castrated rats injected with oestradiol monobenzoate was investigated.

2. In the control animals repeated applications of acetone caused marked cornification of the vaginal epithelium in both virgin and castrated rats but no tumours developed.

3. The vulva responded to the DMBA treatment with marked hyperplasia and subsequent appearance of papillomas and carcinomas. This result was not affected by the presence or absence of the ovary or by the injection of oestradiol to castrated rats.

4. After DMBA-painting the vaginal epithelium showed marked cornification and some hyperplasia in all the rats though not to the same degree as the vulva. Hyperplasia was greatest in the virgin rats, least in the castrated rats and intermediate in the castrated animals given oestradiol. Only one papilloma and one carcinoma were found in 2 of the virgin rats.

5. The DMBA treatment induced sarcomas of the vaginal stroma in 16 of the 22 virgin rats, in 3 of the 13 castrated rats and in 5 of the 16 castrated rats given oestradiol. In addition 3 rats of the last group had pre-sarcomatous lesions.

6. The histogenesis of sarcomas and their relation to focal perivascular infiltration is described and the influence of the ovary and of oestradiol on the

width of the uterine and vaginal stroma and on the induction of sarcomas is discussed.

The authors have pleasure in acknowledging their gratitude to Dr. H. B. Fell, F.R.S. and Dr. F. G. Spear for their constructive criticisms of the manuscript, and to Mr. G. C. Lenney for the photomicrographs and graphs.

REFERENCES

BOAG, J. W. AND GLUCKSMANN, A.—(1956) "Production of cancers in rats by the local application of β-rays and of chemical carcinogens". Contrib. to 'Progress in Radiobiology'. Edinburgh (Oliver and Boyd), p. 476.

GARDNER, W. U.—(1953) "Hormonal aspects of experimental tumorigenesis", in 'Advances in Cancer Research', Vol. 1. New York (Academic Press), 173–232.

Idem AND PAN, S. C.—(1948) *Cancer Res.*, **8**, 241.

GLUCKSMANN, A. AND CHERRY, C. P.—(1956) *Cancer*, **9**, 971.

VON HAAM, E. AND SCARPELLI, D. G.—(1955) *Cancer Res.*, **15**, 449.

HOCH-LIGETI, C.—(1957) *J. nat. Canc. Inst.*, **18**, 661.

MCEUEN, C. S.—(1938) *Amer. J. Canc.*, **34**, 184.

MURPHY, E. D.—(1953) *Amer. J. Path.*, **29**, 608.

PAN, S. C. AND GARDNER, W. U.—(1948*a*) *Cancer Res.*, **8**, 337.

Iidem.—(1948*b*) *Ibid.*, **8**, 613.

PFEIFFER, C. A.—(1949) *Ibid.*, **9**, 277.

REAGAN, J. W., WENTZ, W. B. AND MACHICAO, N.—(1955) *Arch. Path.*, **60**, 451.

VELLIOS, F. AND GRIFFIN, J.—(1957) *Cancer Res.*, **17**, 364.

WOLBACH, S. B.—(1951) *Ann. N.Y. Acad. Sci.*, **53**, 517.

A BIOLOGICAL STUDY OF STRAIN L(P) AND ITS RESPONSE TO 20-METHYLCHOLANTHRENE TREATMENT

K. J. RANADIVE AND S. A. HAKIM

From the Department of Experimental Biology, Indian Cancer Research Centre, Parel, Bombay

Received for publication November 8, 1957

PRELIMINARY studies in chemical carcinogenesis in our laboratories have shown interesting differences in the response of certain inbred strains of mice to 20-methylcholanthrene treatment (unpublished data). These observations have led us to undertake further studies on newer strains of mice. The present paper reports the findings in a new strain, L(P), the first part presenting biological studies on normal animals and the second part reporting observations on their response to 20-methylcholanthrene treatment.

Origin of the strain

The breeding stock of strain L(P) was imported in 1948 from Laboratoire Pasteur de L'Institut du Radium, Paris, and has been maintained here for the last eight years by strict brother-sister mating. In Paris, the strain was originally started in the year 1940 from a hybrid female and was designated L(C). The hybrid female was the offspring of a cross between a female from the cancerous strain XIX (started in 1927, breast cancer incidence 50–70 per cent), and a male of strain XLIII (started in 1932, non-breast cancerous). Neither strain XIX nor strain XLIII was bred by brother-sister mating. At the Pasteur Laboratory the strain L(C) was reported to have a spontaneous breast tumour incidence of 26 per cent in the virgins and 55 per cent in the breeders. Leukaemia was present in about 10 per cent of the animals. Three spontaneous tumours tested at the laboratory of origin were found to possess the milk agent (Rudali, personal communication).

Four females of the L(C) strain were imported after 17 generations of inbreeding in Paris. These females had three pregnancies each and died between the age of 18–24 months without developing either mammary tumours or leukaemia. The inbred strain raised from this stock has not shown either type of spontaneous lesion during the last 8 years. The original strain known as "L(C)" *i.e.* "L" (cancerous) is therefore designated here as "L(P)" *i.e.* "L" (imported from Paris). The line imported in its 17th generation has passed through 14 generations of inbreeding in India.

L(P) mice are albino, with reddish-pink eyes, and have a normal life-span of about 2½ years. The average weight of the adult virgin mouse is 25–30 g. The female reaches sexual maturity at 2½ months with the onset of the first oestrous cycle. The vagina opens between the 50th and 70th day, and the mice start breeding at 2½ months. Vaginal smears studied for periods of 15 days, at monthly intervals, revealed that the regular sex cycle lasts for 6–7 days with a fairly long oestrus up to the age of 6 months. As the females become older, the dioestrus lengthens and the oestrus becomes shorter.

The females are prolific breeders with a long breeding span and a large litter size, generally between six and ten, but litters of twelve are quite common. With advancing age the females tend to obesity.

The mice are kept in an air-conditioned room at a temperature of 76°–78° F., and are fed on a complete diet, analysed for optimum nutritive value, together with water *ad libitum*.

The young are weaned at 21 days and brothers are separated from sisters at 6 weeks. Two females and a male are placed for mating in a breeding cage at 2½ months and pregnant females are isolated in special wooden cages before delivery. On weaning the litter, the females are put back into the mating cage.

MATERIALS AND METHODS

The mice used in the present study belonged to the 10th–14th generations of inbreeding in India. For the biological study of the strain, groups of virgin mice were killed at age periods of 3–4, 5–6, 7–8, 10–12, and over 12 months. Groups of young and old breeders were also included in the study. Sixteen virgins and fourteen breeders were observed until death for the development of tumours, the majority (23/30) being 20–31 months old.

A short experiment was carried out to test for the milk factor in the strain L(P). Since no spontaneous breast tumour was available, it was not possible to test the cell-free tumour extract by the standard method. Pullinger's short-term method (personal communication) was therefore used as follows: eight 2-months-old L(P) virgins were given a single painting of 400 γ of oestradiol in acetone on the dorsal region of the body. Two months later the animals were killed and the mammary glands and ovaries were studied.

Whole mounts of mammary glands fixed in 10 per cent formalin were prepared according to the technique described by Gardner *et al.* (1939) and stained with Mayer's haematoxylin. The adrenals and ovaries were weighed in saline and fixed in Telly's fixative. Serial sections were cut at 6 μ, stained with Mayer's haematoxylin and eosin, and studied by the method described by Taylor and Waltman (1940) and by Fekete (1946, 1953). For the granulosa cell hyperplasia observed in the ovaries the classification of Howell, Marchant and Orr (1954) was used. The mammary tumours were fixed in Bouin's fluid and studied according to the classification of Andervont and Dunn (1949–50), Cloudman (1941), and Bonser (1954). The classification of Khanolkar and Ranadive (1947) was followed for the breast pattern, adding two more acinar types, D and D′, in order to describe the breast state in the breeders. The additional acinar types are:

Acinar type D.—Profuse acinar development, uniform all along the mammary ducts, completely masking the entire ductal system.

Acinar type D′.—A variant of type D, in which a number of huge hyperplastic nodules, some of which may be keratinized, are added to the uniform acinar budding.

Biological Study of the Strain L(P)—(Table I)

Breast pattern

In both the virgins and breeders there was an extensive duct system (Fig. 1, 3). Acinar development was observed in only seven (two virgins and five breeders) of the total ninety-one females studied (Table I). A complete post-

TABLE I.—*Biological Study of Strain L(P)*

Age months	Type of female	Number of animals	Mammary gland: Ductal pattern α	β	β′	γ	Mammary gland: Acinar type A	B	C	C′	Ovaries: Average weight per ovary (mg.)	Average number of corpora lutea per ovary	Number of animals showing granulosa cell hyperplasia	Adrenals: Average weight per adrenal (mg.)	Adrenal histology
3–4	Virgin	12	10	1	—	1	12	—	—	—	2·5	2·6	0/12	1·8	Normal.
5–6	„	12	11	—	—	1	12	—	—	—	2·2	4·0	0/12	2·2	„
7–8	„	10	4	5	—	—	9	—	—	—	3·7	1·2	1/10	2·9	„
10–12	„	13	12	1	—	—	13	—	—	—	2·8	1·1	6/13	2·5	„
12 and over (observed till death)	„	16	10	2	—	2	12	1	—	1	6·0	0	3/16	4·2	„
13–23		3 } 16													
24–31		13 }													
7–10	Breeder	6	—	6	—	—	6	—	—	—	6·3	3·4	1/6	2·1	„
10–12	„	8	5	3	—	—	7	1	—	—	5·0	3·4	1/8	5·6	„
12 and over (observed till death)	„	14	5	9	—	—	10	2	2	—	2·0*	1·3	1/14	3·9	„
16–23		9 } 14													
24–31		5 }													

* = Weight of cystic ovaries not included.

Dutal pattern :
α = Slender ducts, few side branches.
β = Thin ducts with profuse side branches.
γ = Dilated ducts and short blunt off-shoots.

Acinar Types :
A = Acini absent.
B = Acinar groups of 3 to 4.
C = Focal acinar clusters.
C′ = Combination of B and C.

lactation involution of mammary glands was observed in the breeder. The luxuriant acinar proliferation characteristic of breeders of cancer susceptible strains, was never observed in strain L(P).

In the eight virgins which were painted with oestradiol the breast pattern was αA, without any acinar development, suggesting that the milk agent was not present in these mice (Pullinger, 1947).

Ovary

In the normal virgins, the number of corpora lutea was small, the old virgins showing a complete absence of well-formed corpora lutea. A number of normal primary and secondary follicles were present in the ovaries up to the age of 7 to 8 months (Fig. 2). In virgins 10–12 months old, very few well-formed corpora lutea and follicles were present. Diffuse luteinization was seen in the majority of the females and in six of them there was early granulosa cell hyperplasia. In the old virgins there was complete absence of properly developed corpora lutea. A few normal and atretic follicles were present but the majority showed diffuse luteinization. Early granulosa cell hyperplasia was observed in three of sixteen old virgins.

In breeders 7–10 and 10–12 months old, a number of normal and anovular follicles and few well-formed corpora lutea were present (Fig. 4). In old breeders the ovaries were enlarged by the presence of cysts. Only a few normal and atretic follicles were seen, together with an occasional well-formed corpus luteum. Diffuse luteinization was seen in the ovaries of all the old breeders.

The average weight of the ovaries in the oestradiol-treated group was 4·3 mg., and the average number of corpora lutea was 5·6.

Adrenal

These organs showed an increase in weight with age in virgins and had a normal histological structure.

Response of Strain L(P) to 20-Methylcholanthrene Treatment (Table II)

To investigate the role of 20-methylcholanthrene in mammary carcinogenesis and to study the response of the mammary glands and the endocrine organs to the continuous action of the chemical, twenty-seven females of the strain L(P) received cutaneous application of an 0·25 per cent solution of 20-methylcholanthrene in thiophene-free benzene twice a week. Painting was started at 2 months of age and continued till death, painting being continued during both pregnancy and lactation. The site was changed so as to avoid the development of skin tumours. The animals were killed at the time of development of breast or skin tumours. Of twenty-seven females, eight were maintained as virgins, six as normal breeders and the remaining thirteen as forced breeders. Mice of the two latter groups were killed during post-lactation involution. Two of the mice in the forced breeding group, though mated, did not breed and are reported separately.

A sample progeny consisting of ten females and four males, born of six 20-methylcholanthrene-painted breeders, was observed till death.

20-Methylcholanthrene pellets were implanted subcutaneously in nine females which were maintained as forced breeders. The pellets were of paraffin wax,

TABLE II.—*Response of Strain L(P) to 20-Methylcholanthrene Treatment*

Type of female (Number)	Average number of pregnancies	Age at death in months	Number of animals with breast tumours	Ovaries: Average weight per ovary (mg.)	Ovaries: Average number of corpora lutea per ovary	Ovaries: Number of animals showing granulosa cell hyperplasia	Adrenals: Average weight per adrenal (mg.)	Adrenal histology
Control virgins (10)	—	7–8	0/10	3·7	1·2	1/10	2·9	Normal
Painted virgins (8)	—	7–8	0/8	1·9	0·4	0/8	3·7	,,
Control breeders (6)	3·00	7–10	0/6	6·3	3·4	1/6	2·1	,,
Control breeders (8)	2·81	10–12	0/8	5·0	3·4	1/8	5·6	,,
Painted breeders (6)	3·00	7–10	4/6	3·4	5·0	4/6	2·6	,,
Painted forced breeders (11)	3·91	7–10	6/11	4·5	4·6	4/11	2·7	,,
Painted females (mated) which did not breed (2)	—	9	2/2	1·5	One with diffuse luteinization 2nd 6·5	0/2	3·3	,,
Forced breeders with pellets (9)	3·77	6–10	5/9	4·0	3·9	5/9	2·9	,,

the average weight of the pellet being 0·6 mg., and each pellet containing 0·1 mg. of 20-methylcholanthrene. These were placed in contact with the fifth pair of mammary glands, two pellets per animal, one on either side.

EXPLANATION OF PLATES.

FIG. 1.—Portion of mammary gland whole mount of 7-month-old control virgin showing slender mammary ducts with no acinar development (α A). × 17.

FIG. 2.—Section of ovary of 7-month-old control virgin showing large number of Graafian follicles but hardly any corpora lutea. × 17.

FIG. 3.—Portion of mammary gland whole mount of 11-month-old control breeder showing profuse ductal branching without any acini (β A). × 17.

FIG. 4.—Section of ovary of 11-month-old control breeder showing few corpora lutea. × 17.

FIG. 5.—Portion of mammary gland whole mount of 10-month-old breeder treated with 20-methylcholanthrene for 8 months showing profuse ductal branching, uniform acinar buds and hyperplastic nodules (β' D'). × 17.

FIG. 6.—Section of ovary of 10-month-old breeder treated with 20-methylcholanthrene for 8 months, showing large number of corpora lutea. × 17.

FIG. 7.—Portion of mammary gland whole mount of 9-month-old female, which did not breed; treated with 20-methylcholanthrene for 7 months. The mammary gland shows slender mammary ducts with small acinar buds and an occasional focal acinar cluster (α C'). × 15.

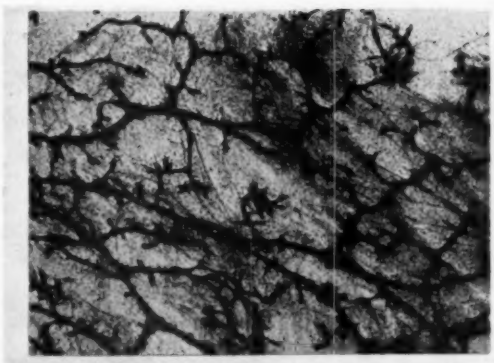
FIG. 8.—Section of ovary of 9-month-old-female which did not breed, treated with 20-methylcholanthrene for 7 months, showing 6 corpora lutea. × 15.

FIG. 9.—Section of non-palpable tumour of the breast of an 8-month-old breeder treated with 20-methylcholanthrene for 6 months. It is an irregular tubular adenocarcinoma with scanty stroma. × 190.

FIG. 10.—Section of palpable breast tumour in an 8-month-old breeder treated with 20-methylcholanthrene for 6 months. It is a small tubular adenocarcinoma of the papillary type. × 190.

FIG. 11.—Section of non-palpable breast tumour in an 8-month-old breeder treated with 20-methylcholanthrene for 6 months. It is an adenoacanthoma showing squamous metaplasia. × 190.

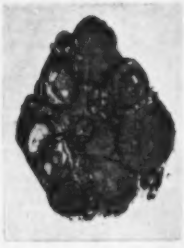
FIG. 12.—Section of a palpable tumour in a 6-month-old breeder treated with 20-methylcholanthrene for 4 months. It is a carcinosarcoma. × 190.



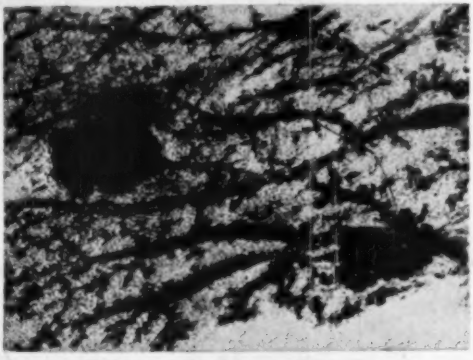
1

2

3

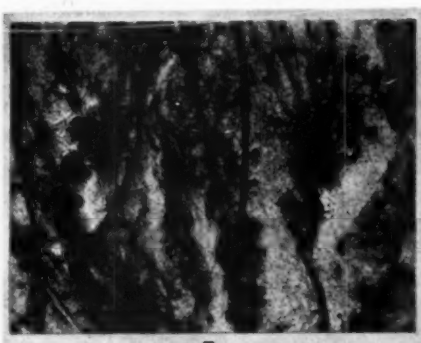


4



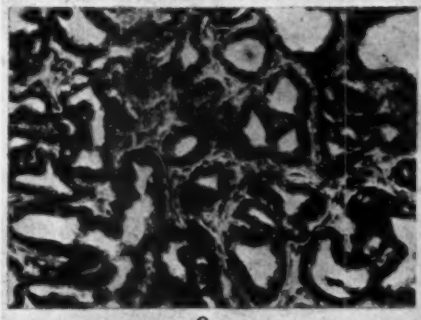
5

6

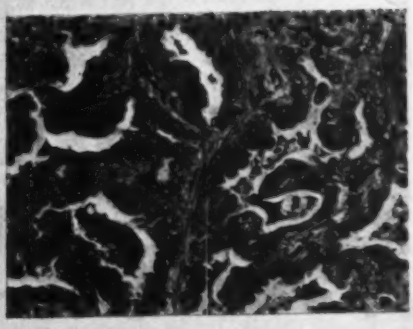


7

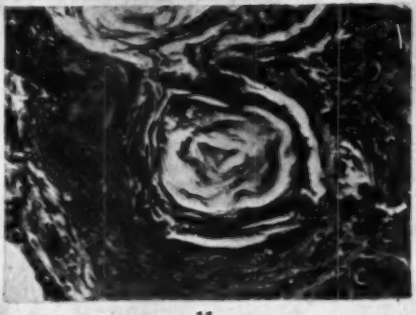
8



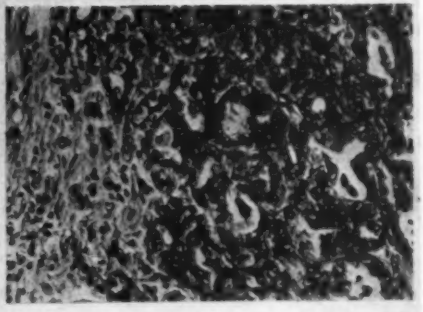
9



10



11



12

EXPERIMENTAL RESULTS

Cutaneous Application of 20-Methylcholanthrene

Breast pattern

(i) *Virgins.*—As in the non-painted control virgin group described above, the 20-methylcholanthrene-treated virgins showed a simple αA mammary pattern without any acinar formation.

(ii) *Breeders.*—In contrast with the single one of the fourteen control breeders which showed acinar proliferation in the breasts (Table I), all the six 20-methylcholanthrene-treated normal breeders presented good duct development and acinar proliferation. Two of these six females showed the D′ type of acinar development (Fig. 5).

(iii) *Forced breeders.*—The mammary ducts were profusely studded with acinar buds, large and small. In six there were keratinized hyperplastic nodules in the mammary glands.

(iv) *Sterile females.*—Two females receiving 20-methylcholanthrene treatment did not breed but showed good ductal development and acinar proliferation in the breasts (Fig. 7).

Breast tumours

These have been divided into two types: those which were palpable in life and those which were not discovered until the skin had been reflected at post mortem (*i.e.* non-palpable tumours). From Table III it is seen that both types of tumour occurred whether the carcinogen was applied to the skin by painting or to the subcutaneous tissues by the insertion of a pellet.

Histology

If all the 40 tumours are considered together, it is apparent that squamous metaplasia was more common in the non-palpable than in the palpable tumours (1 out of 12 palpable compared with 11 out of 28 non-palpable). Apart from this difference, the tumour types were distributed at random throughout the various groups of mice (Table III).

Incidence

(1) *Virgins.*—No mammary tumours occurred in eight virgins surviving for 7–8 months, *i.e.* painted for 5–6 months.

(2) *Breeders.*—Four out of six breeders dying at 7–10 months of age bore mammary tumours. One mouse (P138) bore 12 non-palpable tumours, of which 4 were keratinizing; another mouse bore a single non-palpable tumour; and the remaining two bore palpable tumours.

(3) *Forced breeders.*—Six out of eleven dying at 7–10 months of age bore mammary tumours. In only two mice were the tumours palpable, those in the remaining mice, whether single or multiple, being non-palpable.

(4) *Sterile females.*—These two mice both bore non-palpable tumours.

Ovary

(i) *Virgins.*—In control virgins a small number of corpora lutea were present, together with normal and atretic follicles. The 20-methylcholanthrene-treated

TABLE III.—*Analysis of Palpable and Non-palpable Tumour Types and Structure of Ovaries in Tumour-bearing L(P) Mice*

20-Methyl-cholan-threne-treated females	Animal number	Age at death in months	Classification of mammary tumours								Ovaries		
			Adenocarcinoma			Solid polygonal cell carcinoma	Intratubular carcinoma	Adenoacanthoma	Carcinoma simplex	Carcinosarcoma	Combined weight of both the ovaries (mg.)	Sum of corpora lutea in both the ovaries	Granulosa cell hyperplasia
			Small tubular	Papillary cystic	Irregular tubular								
Breeders . .	P 138	8·4	—	(8)	—	—	—	(4)	—	—	3·8	5	Pseudofollicular.
	P 147	9·8	1	—	—	—	—	—	—	—	3·2	4	—
	P 151	9·10	—	—	—	1	—	(1)1	—	—	5·8	9	Cribriform.
	P 166	10·6	(1)	—	—	—	—	—	—	—	—	19	Pseudofollicular.
Forced breeders	P 146	8·13	—	—	(1)	—	—	—	—	—	2·8	9	—
	P 142	8·15	—	—	—	—	—	(1)	—	—	11·9	14	—
	P 149	8·16	—	1	1	—	—	—	—	—	1·7	9	—
	P 173	8·18	1	—	(1)	—	1	(1)	(1)	—	12·9	13	—
	P 170	9·10	(1)	—	—	—	—	(1)	—	—	27·9	11	Tubular adenoma, follicular cyst.
	P 171	10·3	(1)	—	—	—	—	(1)	—	—	9·5	9	Pseudofollicular.
Mated females which did not breed	P 155	9·4	—	—	—	—	—	(1)	—	—	4·7	13	—
	P 156	9·4	—	—	(1)	—	—	—	(1)	—	0·9	0	Diffuse luteinization.
Forced breeders with pellets	P 218	6·13	—	—	—	—	—	—	—	1	8·2	9	Pseudofollicular.
	P 302	9·4	—	—	—	—	—	—	—	1	5·8	6	„
	P 251	9·9	—	—	1	—	—	—	—	—	10·4	4	„
	P 257	9·23	—	2	—	—	—	—	—	—	9·0	7	Diffuse luteinization.
	P 324	10·29	(1)	—	—	—	—	(1)	—	—	8·8	10	Pseudofollicular.
Total .	17	—	6	11	5	1	1	12	2	2	8·00	8·9	11/17

Figures in the bracket represent non-palpable lesions.

virgins also showed very few corpora lutea but a number of normal and anovular follicles. Granulosa cell hyperplasia was not seen in these females.

(ii) *Normal breeders.*—Unfortunately only a small number of normal control breeders of the same age-group was available for comparison. In six breeders, 7–10 months old and eight 10–12 months old the average number of corpora lutea was 3·4. The ovaries of the 20-methylcholanthrene-treated breeders of 7–10 months contained an average of 5·0 corpora lutea and many normal and anovular follicles (Fig. 6). Granulosa cell hyperplasia was observed in four of the six females.

(iii) *Forced breeders.*—The treated forced breeders developed on an average 4·6 well-formed corpora lutea. Granulosa cell hyperplasia was present in the ovaries of four females. No normal forced bred controls were maintained.

(iv) *Sterile females.*—Of the two females that failed to breed, one presented extensive diffuse luteinization in the ovaries, while in the other there were 6 to 7 functional corpora lutea (Fig. 8).

Adrenal.—The adrenals from all the groups, *i.e.* control and treated virgins, breeders and sterile females presented a normal histological picture. 20-Methylcholanthrene did not have any effect on the structure of the adrenal.

Subcutaneous Implantation of Pellets Containing 20-Methylcholanthrene

Breast pattern

All the nine forced breeders implanted with 20-methylcholanthrene pellets showed good duct and acinar development.

Breast tumours

Seven of the nine forced breeders developed palpable tumours at the site of implantation of the pellets. With the exception of two fibrosarcomas of the subcutis the remainder were either adenocarcinomas, adenocanthomas or carcinosarcomas (Fig. 12).

Seven females showed tumours at the site of the pellet, two having multiple tumours. Two were fibrosarcomas of the subcutis, two were sarcomas with foci of tubular adenocarcinoma, one was a carcinosarcoma and three were adenocarcinomas of the mammary gland. One female showed two tumours which were not palpable and were classified as adenoacanthoma and adenocarcinoma respectively.

Ovary

These forced breeders showed a number of well-formed corpora lutea together with normal and anovular follicles. Early granulosa cell hyperplasia was seen in five of the nine females.

Adrenals

All the adrenals showed normal histology.

Progeny

Table IV compares the breeding behaviour and survival of litters of both control and 20-methylcholanthrene-treated breeders. The mortality was low in the progeny of the control as well as of the painted normal breeders.

Of ninety-two young born to the six painted breeders, forty-two survived. Ten females and four males from these are at present under observation. Five of these ten females are being maintained as forced breeders and have so far undergone four to six normal pregnancies. The remaining five females are virgins. The males appear normal and healthy.

TABLE IV.—*Breeding Behaviour of Control and 20-Methylcholanthrene-treated Female Parents*

Type of parent	Total number of parents studied	Average number of pregnancies	Total number of young born	Average size of litter
Control breeders	28	2·90	513	6·33
Painted breeders	6	3·00	92	5·16
Painted forced breeders	11	3·91	250	5·81
Forced breeders with pellets	9	3·77	195	5·73

DISCUSSION

The albino strain L(P) is derived from a strain maintained in Paris designated L(C). This latter strain yielded a moderate incidence of mammary tumours in both virgins and breeders, and was thought to carry the milk factor. No mammary tumours have yet been observed in the L(P) strain among 29 virgins and 22 breeders ranging in age from 10–31 months (Table I). As no spontaneous mammary tumours were available for testing for the milk factor, an attempt was made to demonstrate acinar growth following a single application of oestradiol to the skin. This was unsuccessful, which suggests that the milk factor is not present (Pullinger, 1947).

A study of the breast structure of the L(P) strain showed that the duct system was well developed in virgins and breeders, but that acini were singularly deficient in virgins and disappeared from the breeders immediately after lactation. It was noted also that the number of corpora lutea in the ovary was of a low order, the largest number being observed in virgins of 5–6 months of age. In both virgins and breeders corpora lutea were deficient after 12 months of age. It is suggested that there is a causal relationship between the poorly developed breast and the low corpus luteum content of the ovary.

Assuming that this strain is a low breast cancer strain with a poorly developed breast, it seemed valuable to know whether tumours could be induced by chemical means. Two methods of application of 20-methylcholanthrene were used: continuous skin painting in benzene and subcutaneous implantation in paraffin wax. Both methods were successful in inducing tumours. A good yield was obtained in painted breeders, forced breeders and sterile mated females, as well as in implanted forced breeders. No tumours were obtained in eight continuously painted virgins surviving to 8 months of age. This result contrasts with that of Bonser (1954), who found that subcutaneous implantation of this chemical even for a limited period of time induced mammary tumours in IF virgins, and with those of Orr (1951) and Jull (1954), who found that skin applications of 20-methylcholanthrene in oil for a limited period was equally successful in inducing tumours in IF virgins. Marchant (1955), on the other hand, showed that pregnancy and lactation lowered the tumour yield in response to oily skin applications of 20-

methylcholanthrene to IF mice. An analysis of the number of corpora lutea (Table II) in control and carcinogen-treated mice showed that there was a tendency towards a higher corpus luteum content in the treated groups. As the corpus luteum content of the ovaries of non-treated forced breeders is not known, the question of the role played by the ovary in the chemical induction of the mammary tumours must remain uncertain. If further work should show that the corpora lutea are stimulated to function by the chemical, then it might well be that there is a causal relationship between the increased number of corpora lutea and the development of the mammary tumours.

The mammary tumours resulting from the methylcholanthrene treatment were of the usual varied type which is to be expected in chemically induced tumours. Twelve of the tumours were of a size which caused visible and palpable lumps, whereas the remaining 28 were only discovered at post mortem. There was a higher incidence of squamous change in the latter group, possibly due to their slower growth rate. There was no doubt that they were true tumours (Fig. 9, 11).

SUMMARY

A new strain of albino mice, designated L(P), is described. It was derived from a Parisian strain inbred for 17 generations, and has been maintained in India during 14 generations of inbreeding. The breast of the virgin female has a well-developed duct system but only a poor acinar system ; acini disappear from breeders soon after lactation. Spontaneous mammary tumours have not been observed in 39 virgins and 28 breeders ranging in age from 7–31 months. The average number of corpora lutea per ovary is low, especially after the age of 6 months in virgins and 12 months in breeders.

Virgins and breeders were subjected to two forms of treatment by 20-methylcholanthrene : continuous cutaneous application of an 0·25 per cent benzene solution and subcutaneous implantation of paraffin wax pellets each containing 0·1 mg. of the carcinogen. Mammary carcinomas were induced by both methods of treatment. No tumours were obtained in 8 virgins dying at 7–8 months of age, but a good yield was obtained in painted breeders, forced breeders and mated females which failed to breed. Acinar development was also improved by the treatment.

20-Methylcholanthrene had no effect on the breeding behaviour of the L(P) mothers nor on the health of the progeny. Litters of normal size and number were born.

Grateful thanks are due to Dr. V. R. Khanolkar, for his encouragement and interest during the course of this work.

REFERENCES

ANDERVONT, H. B. AND DUNN, T. B.—(1949–50) *J. nat. Cancer Inst.*, **10**, 895.

BONSER, G. M.—(1954) *J. Path. Bact.*, **68**, 531.

CLOUDMAN, A. M.—(1941) 'Biology of the Laboratory Mouse'. Philadelphia (The Blakiston Co.), p. 168.

FEKETE, E.—(1946) *Cancer Res.*, **6**, 263.

Idem.—(1953) *Anat. Rec.*, **117**, 93.

GARDNER, W. U., STRONG, L. C. AND SMITH, G. M.—(1939) *Amer. J. Cancer*, **37**, 510.
HOWELL, J. S., MARCHANT, J. AND ORR, J. W.—(1954) *Brit. J. Cancer*, **8**, 635.
JULL, J. W.—(1954) *J. Path. Bact.*, **68**, 547.
KHANOLKAR, V. R. AND RANADIVE, K. J.—*Ibid.*, **59**, 593.
MARCHANT, J.—(1955) *Ibid.*, **70**, 415.
ORR, J. W.—(1951) *Acta Un. int. Cancr.*, **1**, 294.
PULLINGER, B. D.—(1947) *Brit. J. Cancer*, **1**, 177.
TAYLOR, H. C. AND WALTMAN, C. A.—(1940) *Arch. Surg.*, **40**, 733.

THE INHIBITORY EFFECT OF CONTINUED LACTATION ON THE INCIDENCE OF CHEMICALLY-INDUCED BREAST TUMOURS IN MICE OF THE IF STRAIN

JUNE MARCHANT

From the Cancer Research Laboratories, Medical School, Birmingham, 15

Received for publication November 8, 1957

In strains of mice in which the mammary tumour agent is present and transmitted from one generation to the next in the mother's milk, a high natural incidence of breast cancer occurs. In most of these strains the incidence of breast cancer is higher amongst breeding females than amongst non-breeders—a situation which seems to be the reverse of the tendency found, though much less strikingly, in women (Versluys, 1955 ; Macdonald, 1942 ; Wainwright, 1931).

The IF strain of mice established by Bonser (1938) does not exhibit spontaneous breast tumours, but its breast tissue is particularly sensitive to the carcinogenic action of methylcholanthrene applied either locally (Bonser, 1954) or at a distance (Orr, 1943 and 1946). The breast tumours thus induced have been shown to lack the mammary tumour agent (Dmochowski and Orr, 1949).

In a preliminary report in 1955, Marchant showed that continued breeding with full lactation reduced the incidence of methylcholanthrene-induced breast tumours in IF and IF hybrid mice, and they were prevented from forming when lactation occurred before the first treatment with the carcinogen. These studies have now been extended in an attempt to find the essential factor in preventing the induction of breast tumours in this strain.

MATERIALS, METHOD AND RESULTS

All the mice used in these experiments were females of the IF strain. The standard carcinogenic treatment they received was a total of 8 applications at fortnightly intervals of a solution of 0·5 per cent 20-methylcholanthrene (MC) in olive oil. An average dose of 0·20 ml. = 1 mg. of methylcholanthrene was applied in 16 drops, 4 on each side of the dorsal and ventral surfaces. Two groups of mice received similar treatment with 0·5 per cent 9 : 10-dimethyl-1 : 2-benzanthracene (DMB) in olive oil, which also induces breast tumours in IF mice (Howell, Marchant and Orr, 1954).

For this report the presence of mammary tumours was judged clinically and confirmed by histological study of the tumours. In most cases the ovaries of the mice were examined histologically. Mice not surviving the full period of carcinogenic treatment or not coming to necropsy are excluded from the results.

1. *The Incidence of Breast Tumours in Virgin and Breeding IF Mice Treated with Dimethylbenzanthracene (DMB) Compared with Those Treated with Methylcholanthrene (MC)*

The effect of DMB treatment on virgin and breeding IF females was studied for comparison with the original observations using MC as the carcinogen. The

virgin females received the first of their 8 fortnightly treatments with DMB at 1½ to 4 months of age. Fifteen breeding mice were mated individually to untreated male IF mice and allowed to suckle their young. The first treatment with DMB was given *after* the birth of the first litter, when the mice were 3 to 3¾ months of age. Litters were removed when the young were 3 to 4 weeks old.

Results

In Table I the incidence of breast tumours in the animals coming to necropsy is given and compared with that previously obtained with MC.

TABLE 1.—*Effect of Natural Breeding on Induction of Breast Tumours in IF Mice by Methylcholanthrene and Dimethylbenzanthracene Treatment begun after Lactation had Commenced.*

Mice	Carcinogen	Age in months at 1st painting (range)	Survival in months from 1st painting (range)	Mean number of litters suckled after 1st painting (range)	Number of mice with breast tumours (muitiple)	Incidence of mice with breast tumours (per cent)	Number of mice with ovarian tumours or lesions (macroscopic)
43 Virgin	DMB	2·7 (1·5–4)	6·7 (5–8·5)	0	33 (19)	77	24 (9)
19 Virgin	MC	2·7 (2·3–3)	10·0 (6·5–19)	0	14 (2)	74	1 (0)
9 Breeders	DMB	3·3 (3–3·8)	5·9 (4·5–6)	2·0 (1–3)	2 (0)	22	2 (0)
12 Breeders	MC	4·3 (3·8–5·3)	10·1 (6·5–16)	4·7 (2–6)	0 —	0	0 —

It can be seen that breeding markedly reduced the incidence of breast tumours in IF mice treated with DMB (from 77 per cent to 22 per cent) just as in those treated with MC (from 74 per cent to 0). In the case of DMB a χ^2 test shows the difference between the incidence in breeders and virgins is statistically significant $P < 0{\cdot}05$).

Although the incidence of breast tumours in virgin IF mice was similar with both carcinogens it can be seen that there was a significant difference in the survival of the virgins treated with DMB and those treated with MC ($P < 0{\cdot}001$). Survival of the breeders treated with these carcinogens was of the same order as that of virgins treated with the same carcinogen.

It can also be seen from Table I that DMB has a much greater power to induce neoplastic changes in the ovaries of IF mice than MC.

When a histological comparison was made of the ovaries of normal and breeding mice treated with DMB or MC for 5 to 6 months, the essential difference in their action could be seen. In the ovaries of virgins and breeders treated with DMB no oocytes or developing follicles could be found, whereas in those of mice treated with MC they could still be seen. This destructive effect of DMB on oocytes also occurs in other strains than IF (Marchant, 1957). It is reflected in the comparison of the breeding history of the IF mice treated with it and those treated with MC in Table II.

The mean number of litters suckled by the mice after treatment with MC was twice as many as the number suckled after DMB treatment. The number of mice treated with MC and producing more than 2 subsequent litters was signifi-

TABLE II.—*Comparison of MC and DMB Treatment on Breeding History of IF Female Mice*

Number of mice*	Carcinogen	Number of litters suckled before 1st painting	Mean number of litters suckled after 1st painting (range)	Period from first painting to birth of last litter (months)
18	MC	1	3·8 (1–7)	7·5
15	DMB	1 (2 in 3 cases)	1·9 (0–3)	2·3

* The number of mice given here is larger than that given in Table I since it includes some which were not autopsied for various reasons.

cantly greater than those treated with DMB and producing more than 2 litters. Using a χ^2 test, $P < 0{\cdot}01$. The mouse which remained fertile longest after DMB treatment produced its last litter 2·3 months after the first DMB treatment, while with MC the period was 7·5 months.

Histologically the breast tumours induced by DMB were similar to those induced by MC and have been previously described by Orr (1951). One point of interest was that in the virgins treated with MC only 2 of the 14 mice with breast tumours showed any evidence of squamous metaplasia in the tumours, but in the virgins treated with DMB 17 of the 24 mice with tumours showed squamous metaplasia.

2. *Incidence of Breast Tumours in Breeding IF Mice after Intranasal Administration of Methylcholanthrene*

It was considered possible that in the breeding female mice the effectiveness of the carcinogen might have been lost by the spreading of some of the oily vehicle over the mate and the young. It was therefore decided to administer the solution of the carcinogen intranasally, since Orr (1943) found it to be an effective method of inducing breast tumours in this strain. Accordingly, after the first litter had been born, the mice were deeply anaesthetised with ether. When the breathing had become rapid and regular, olive oil containing 0·5 per cent MC was dropped from a pipette over the nostrils. Several drops could be administered in this way. The procedure was repeated at fortnightly intervals except when a mouse was in the last few days of pregnancy. After 8 treatments in all, the mice were kept mated until death.

Results

In the 7 mice which survived the full carcinogenic treatment, no breast tumours occurred. The mean survival of the mice from first treatment was 10·5 months (range 4·5 to 14·5). The mean number of litters produced after treatment was 2·4 (range 0 to 4), which was not so great as in the MC painting experiment. The period from the first MC inhalation to the birth of the last litter was 13 months, considerably longer than the 7·5 months in the painting experiment. In fact, these mice seemed to become sterile after one or two litters had been suckled, but in four cases they began to breed again 3 or 4 months after the last MC treatment.

The absence of breast tumours in these breeding mice contrasts strongly with the incidence of 69 per cent in virgin IF mice subjected to intranasal administration of MC (Orr, 1943).

3. *The Effect of Varying the Conditions of Breeding on the Induction of Breast Tumours in IF Mice by Methylcholanthrene*

Having established that continued breeding with full lactation reduced the incidence of breast tumours in IF mice after carcinogen treatment from about 75 per cent to virtually none, several experiments were performed to try to establish the essential factors responsible. Four groups of mice will be reported here.

The first group of females were forced bred, that is they were allowed to breed freely but not to suckle their young. The latter were removed from the mothers as soon as they were discovered. The first MC treatment was given after the birth of the first litter to 9 mice and at mating to 4 mice.

A second group of females were kept in a state of pseudo-pregnancy by mating them individually to vasectomised males of the age of 2·5 months. MC paintings were begun at the age of 5 months.

In a third group of mice breeding and lactation were permitted but the carcinogen treatment was commenced at mating, i.e. before the first lactation.

A fourth group were allowed to suckle one litter only before carcinogen treatment commenced. No further litters were allowed, the males being removed.

Results

The incidence of breast tumours occurring in these mice is shown in Table III.

TABLE III.—*The Effect of Varying the Conditions of Breeding on the Incidence of Breast Tumours Occurring in IF Mice Treated with Methylcholanthrene*

Mice	Treatment	Age in months at 1st painting (range)	Survival in months from 1st painting (range)	Number of mice with breast tumours (multiple)	Incidence of mice with breast tumours (per cent)
4	Forced breeding, MC before 1st litter born	6·0 (5·3–6·3)	5·4 (3·8–6·0)	3 (0)	69
9	Forced breeding, MC after 1st litter born	5·9 (4·3–7·8)	5·2 (3·5–6·5)	6 (3)	
9	Pseudo-pregnant	5·0	4·5 (3·5–5·8)	9 (5)	100
7	Natural breeding, MC before 1st lactation	6·6 (6·3–7·3)	5·3 (3·5–6·0)	2 (0)	29
13	One lactation only, breeding stopped, then MC	5·1 (3·3–7·0)	6·2 (4·0–9·0)	9 (6)	70

It will be seen that breast tumours occurred in all the groups of animals. The incidence in the forced breeding group (69 per cent) and in the mice allowed to suckle one litter only before carcinogen treatment (70 per cent) was not significantly different from the incidence obtained in virgins (74 per cent), see Table I. In the natural breeding group given MC before the first lactation the incidence of tumours was lower (29 per cent) than in virgins, but this was not statistically significant ($P = 0·1$) and the tumours were not eliminated altogether,

as when the MC treatment was begun after the first lactation. In the pseudo-pregnant group, on the other hand, an incidence of 100 per cent was obtained, although the survival of this group was slightly less than that of the others. The difference in proportions between the pseudo-pregnant and virgin mice bearing breast tumours is not statistically significant.

The ovaries of the forced breeding group and those of the pseudo-pregnant group showed some large corpora lutea, primordial follicles, few or no large follicles and often a good deal of blood vessel congestion. The mice which were allowed to lactate once, and were then prevented from breeding further, had ovaries which were diffusely luteinised. Atretic follicles, but few or no developing follicles or corpora lutea were present in them. Some showed blood vessel congestion and one of the 13 had a microscopically detected early granulosa-celled tumour.

Table IV shows the breeding history of the breeding mice in the above experiments. It includes some mice which did not come to autopsy.

From this table it can clearly be seen that, when the carcinogenic treatment is begun at mating, fertility is reduced in both forced breeding and natural breeding groups.

TABLE IV.—*Effect of MC Treatment on Forced Breeding and Natural Breeding IF Mice*

Mice	Treatment	Number of litters before 1st painting	Mean number of litters after 1st painting (range)
4	Forced breeding	0	1·5 (1–2)
9	„ „	1	3·9 (2–5)
9	Natural breeding	0	1·6 (1–2)
*18	„ „	1	3·8 (1–7)

* Included from Table II.

DISCUSSION

The results of the first two experiments confirm the fact previously reported (Marchant, 1955) that natural breeding with full lactation significantly lowers the incidence of breast tumours induced by chemical carcinogens in IF mice. Although two of the nine breeders treated with DMB developed breast tumours, these occurred in the only two mice of the group which were subsequently found to have ovarian tumours.

The results shown in Table I suggest that DMB is a more potent breast carcinogen than MC, for although the incidence of breast tumours is the same with both carcinogens, the much shorter survival of the DMB-treated group indicates a more rapid induction of breast tumours with this carcinogen. Another fact in support of this is the higher incidence of multiple tumours in virgin mice after treatment with DMB. There was also a higher proportion of tumours in the DMB-treated mice which showed squamous metaplasia.

Tables I and II show that DMB has a much greater effect on ovaries of IF mice than MC, producing a high incidence of granulosa-cell tumours in virgins

and lowering the fertility of the breeders. Histological comparisons of the ovaries showed the essential difference was the complete destruction of oocytes by DMB, resulting in a disappearance of follicles.

Methylcholanthrene itself apparently has some effect in reducing fertility. Orr (1946) described the failure of 12 IF females to breed after treatment with MC paintings for 86–149 days. Table IV shows that, when carcinogen treatment is begun before breeding, subsequent fertility is impaired. This table also shows that a single pregnancy before carcinogen treatment has a protective effect against subsequent impairment of fertility.

With regard to the lowering of the incidence of chemically induced breast tumours by natural breeding, it can be seen from the third experiment that repeated lactation is the essential factor. Forced breeding did not result in a lower incidence than in virgins and pseudo-pregnancy actually raised the incidence. A single lactation had no effect in lowering the incidence of breast tumours in mice subsequently treated with MC and prevented from breeding again. This seemed to indicate that repeated lactations during the carcinogen treatment were essential. However, natural breeders given the first carcinogen treatment before breeding produced some breast tumours. To obtain the best protection against breast tumours, it was necessary for the first litter to be suckled before the carcinogen treatment began.

That the protective effects of lactation against the chemical induction of breast tumours do not apply to all strains of mice reputedly free from milk factor, has recently been shown by Ranadive and Hakim (1958). Using the L(P) strain, which had been bred for 8 years in India without producing spontaneous breast tumours, they obtained no breast tumours in virgins treated with MC but a high incidence in natural and forced breeders. It would therefore be of great interest to compare the L(P) and IF strains of mice from an endocrinological point of view.

SUMMARY

1. A high incidence of breast tumours (77 per cent) was induced by painting with dimethylbenzanthracene (DMB) in virgin mice of the IF strain, which is free from the mammary tumour agent.

2. A lower incidence (22 per cent) was induced in naturally-breeding IF mice which were allowed to lactate before DMB treatment began.

3. No breast tumours were induced in 7 naturally breeding IF mice by the inhalation of methylcholanthrene (MC), though they have previously been obtained in 69 per cent of virgins so treated.

4. The incidence of breast tumours obtained after MC painting was as high in forced-bred females (69 per cent) as in virgins previously reported (74 per cent),

5. One lactation only, followed by MC painting and no further breeding, gave a breast tumour incidence of 70 per cent.

6. Naturally-breeding females in which MC paintings were begun before breeding had an incidence of 29 per cent.

7. Pseudo-pregnancy raised the incidence of breast tumours to 100 per cent.

8. The effects of the various procedures used on the histological structure of the ovary and on fertility is discussed.

9. It is concluded that DMB is more potent in its action on breasts and ovaries than MC.

10. The greatest protection against the induction of breast tumours by DMB and MC in IF mice is afforded by continued pregnancies with full lactation, especially if lactation has commenced before the carcinogen is first applied.

I am grateful to Professor J. W. Orr for allowing me to include data from some of his experiments, and to the Birmingham Branch of the British Empire Cancer Campaign for support of this work.

REFERENCES

BONSER, G. M.—(1938) *J. Path. Bact.*, **46**, 581.—(1954) *Ibid.*, **68**, 531.
DMOCHOWSKI, L. AND ORR, J. W.—(1949) *Brit. J. Cancer*, **3**, 520.
HOWELL, J. S., MARCHANT, J. AND ORR, J. W.—(1954) *Ibid.*, **8**, 635.
MACDONALD, I.—(1942) *Surg. Gynec. Obstet.*, **74**, 75.
MARCHANT, J.—(1955) *J. Path. Bact.*, **70**, 415.—(1957) *Brit. J. Cancer* (in press).
ORR, J. W.—(1943) *J. Path. Bact.*, **55**, 483.—(1946) *Ibid.*, **58**, 589.—(1951) *Acta Union. Internat. contre. Cancer*, **7**, 294.
RANADIVE, K. J. AND HAKIM, S. A.—(1958) *Brit. J. Cancer* **12**, 44.
VERSLUYS, J. J.—(1955) *Ibid.*, **9**, 239.
WAINWRIGHT, J. M.—(1931) *Amer. J. Cancer*, **15**, 2610.

THE INDUCTION OF BREAST TUMOURS BY METHYLCHOLANTHRENE IN CASTRATED MALE AND FEMALE MICE OF THE IF STRAIN BEARING OVARIAN GRAFTS

JUNE MARCHANT

From the Cancer Research Laboratories, Medical School, Birmingham, 15

Received for publication December 4, 1957

HUSEBY AND BITTNER (1951) published a study of the development of spontaneous breast tumours in castrated male mice of the A strain bearing ovarian grafts. They found an incidence of 40 per cent developed breast tumours, whereas only 5 per cent had been found to occur in a small group of ovariectomised female mice bearing subcutaneous ovarian grafts (Huseby and Bittner, 1948). Four per cent of virgin and 80 per cent of breeding female mice of this strain develop breast cancer. Loeb, Blumenthal and Kirtz (1944) had also reported a greater frequency of breast tumours in castrate male mice bearing ovarian grafts than in normal females bearing such grafts. Their experiments were done with various strains, but over half were A strain. However, the early work of Murray (1928) suggested that in the dba strain approximately the same low frequency occurred in castrated males with subcutaneous ovaries as in virgin females of the same strain.

The present study was undertaken to compare the incidence of chemically-induced breast tumours in castrated male and female mice of the IF strain bearing ovarian grafts. Unlike the A strain, the IF strain is free from the mammary tumour agent (Dmochowsky and Orr, 1949), but its breast tissue is extremely sensitive to the action of certain chemical carcinogens. Induction of breast tumours in males and females of this sensitive strain has recently been studied in some detail (Jull, 1954 ; Bonser, 1954 ; Marchant, 1958), but no direct comparison of the two sexes has previously been made in this manner.

MATERIALS AND METHODS

The ovaries of 25 female mice of the IF strain were removed at the age of 9–11 weeks and at the same time one ovary from another female IF was implanted subcutaneously in one flank. In 11 cases the grafted ovary was from a litter mate. An IF ovary was implanted under the skin of 27 male IF mice aged 6–24 weeks (75 per cent were under 12 weeks old). None of these grafts were from litter mates. The male mice were castrated at the same time. Between 10 and 20 days after operation, 8 fortnightly paintings of 0·5 per cent methylcholanthrene in olive oil were commenced. The animals were maintained on rat cube (Thompson's formula) with water *ad libitum*. All mice were kept until dead or moribund, or their breast tumours had grown very large. At necropsy breast tumours were counted and removed together with the ovarian transplant for microscopic examination. Only those mice which survived full treatment have been included in this communication.

RESULTS

Tumour development

Approximately the same incidence of breast tumours occurred in the female IF mice bearing an ovarian graft from a litter mate as in those bearing a graft from a more distant relative. These two groups will therefore be considered together in Table I which gives the results.

TABLE I.—*The Induction of Breast Tumours by Methylcholanthrene in Castrated Male and Female Mice of the IF Strain Bearing Ovarian Grafts*

Sex	Number	Number with breast tumours (multiple)	Per cent breast tumours	Mean survival in month from first MC treatment (range)
Male	27	17 (7)	63	7·0 (4–11)
Female	25	19 (6)	76	7·1 (3·5–12·3)

The mean survival of the two groups is almost identical indicating a similar induction time of the tumours.

Histology of ovarian grafts

Ovarian grafts in 7 males and 13 females were examined histologically. There was no noticeable difference in grafts in the two sexes. Mature follicles were found in some of the youngest grafts (up to 5 months). Corpora lutea were not found in grafts older than 6 months. Atretic follicles were absent after about 7 months. After this the grafts consisted of large cells, often pigmented, sometimes with some giant cells and infiltration by lymphocytes. Occasionally tiny cysts or tubules were found. Germinal epithelium was missing from all grafts, which were encompassed by fibrous tissue. No granulosa-cell proliferations were seen except in one graft in a male mouse dying after only 2½ months' treatment and therefore not included in the table of results.

Breast tumour histology

Almost all the breast tumours were examined histologically. They were similar to those described previously in this strain (Orr, 1951; Bonser, 1954). Squamous metaplasia was seen in the breast tumours in 7/17 males and in 12/19 females.

DISCUSSION

The results described above indicate that, in the incidence of breast tumours induced by MC in castrated IF mice bearing ovarian grafts, there is no significant difference between males and females. The incidence is of the same order as that found in virgin females of this strain similarly treated (Orr, 1946—69 per cent; Marchant, 1955—75 per cent) and that found in oestrogen-treated intact males following fortnightly intranasal administrations of MC (Orr, 1943—69 per cent). Normal males given MC intranasally yielded no breast tumours however (Orr, 1943).

The incidence of induced breast tumours in the IF strain in the two sexes reported here contrasts with that described by Huseby and Bittner (1951) for spontaneous breast tumours in the A strain. These authors considered that the greater frequency of breast cancer development which they obtained in castrated male mice bearing ovarian grafts than in castrated females bearing similar grafts might be, at least in part, due to the essentially non-cycling male pituitary maintaining constant stimulation. Clearly such a consideration, if relevant, has no effect on the chemical induction of breast tumours in the IF strain.

SUMMARY

Breast tumours were induced by 8 fortnightly applications of 0·5 per cent methylcholanthrene in olive oil in 17 of 27 (63 per cent) castrated male IF mice bearing a subcutaneous ovarian graft, and in 19 of 25 (76 per cent) ovariectomised females bearing a similar graft. This incidence is similar to that obtained in virgins of this strain.

I am grateful to the Birmingham Branch of the British Empire Cancer Campaign for support of this work.

REFERENCES

Bonser, G. M.—(1954) *J. Path. Bact.*, **68**, 531.
Dmochowsky, L. and Orr, J. W.—(1949) *Brit. J. Cancer*, **3**, 520.
Huseby, R. A. and Bittner, J. J.—(1948) *Acta Un. int. Cancr*, **6**, 197.
Idem .—(1951) *Cancer Res.*, **11**, 450.
Jull, J. W.—(1954) *J. Path. Bact.*, **68**, 547.
Loeb, L., Blumenthal, H. T. and Kirtz, M. M.—(1944) *Science*, **99**, 230.
Marchant, J.—(1955) *J. Path. Bact.*, **70**, 415.
Idem.—(1958) *Brit. J. Cancer*, **12**, 55.
Murray, W. S.—(1928) *J. Cancer Res.*, **12**, 18.
Orr, J. W.—(1943) *J. Path. Bact.*, **55**, 483.
Idem.—(1946) *Ibid.*, **58**, 589.
Idem.—(1951) *Acta Un. int. Cancr.*, **7**, 294.

THE PATHOLOGICAL EFFECTS OF THE INTRAPERITONEAL INJECTION OF 3 : 4-BENZPYRENE INTO RATS AND MICE

S. PAYNE

From the Department of Cancer Research, Mount Vernon Hospital and the Radium Institute, Northwood, Middlesex

Received for publication December 5, 1957

THE experiments described in this paper were designed to show the pathological effects of 3 : 4-benzpyrene when it is injected as an aqueous colloid into the peritoneal cavity of rats and mice. This medium and route have been successfully employed in experiments concerning the metabolism of benzpyrene (Calcutt and Payne, 1953, 1954*a*, *b* and *c*, and 1955), and thus it seemed of some importance to ascertain if any pathological changes were associated with this process.

MATERIALS AND METHODS

In each case the 3 : 4-benzpyrene was injected into the abdominal cavity as an aqueous colloidal suspension prepared in a similar manner to that described by Boyland (1932).

The animals used were male and female mice of the ST. A. strain, and male and female rats of the Wistar strain. The mice were 12 weeks old at the commencement of the experiment and the rats were 6 months old. They were fed Diet 18, and water *ad libitum*. The animals were killed when they became ill, when tumours appeared, or one year after the injection. The mice were killed by breaking the spinal cord, and the rats by a blow on the head. These methods were used to avoid artefacts in the cytology.

A complete post mortem examination was carried out immediately after death. The abdominal and pleural cavities were examined under an ultra-violet lamp fitted with a Wood's glass filter, in order to detect any fluorescence that could be due to the presence of benzpyrene, or its metabolites, in or on any of the organs.

Portions of the liver, spleen, kidney, duodenum, adrenals, genitalia, and of all tumour masses were taken and fixed in Zenker formol. After 18 hours fixation the tissues were washed in running tap water for 12–24 hours, dehydrated in alcohol, and cleared in chloroform prior to embedding in paraffin wax. Sections were cut at 4–5 μ, and for routine histological examination were stained in Ehrlich's acid haematoxylin and aqueous eosin.

Special fixation and staining techniques were employed to demonstrate patricular cytological features. Thus sections of liver were stained by the Bensley-Cowdry method to demonstrate mitochondria, and the Feulgen technique for nuclear detail.

RESULTS

(a) *Mice*

Forty ST. A. male, and 40 ST. A. female mice used in this experiment each received an intraperitoneal injection of 2 mg. of 3 : 4-benzpyrene, contained in 2 c.c. *aq. dest.*

Intra-abdominal tumours occurred in most of the animals, the first appearing 15 weeks after the injection. A slightly higher percentage of tumours occurred in the female mice, *viz.* 81 per cent as compared with 73 per cent in the males, but the average latent period was the same in both sexes, *i.e.* 33 weeks.

At the post mortem examination the abdominal cavity was often found to be full of clear straw-coloured, or blood-stained ascitic fluid. The tumour masses, which were usually large, white, and hard in consistency, occurred mainly in the mesentery between the abdominal organs. In the female mice 70 per cent of the tumours were situated between the spleen and stomach, but in the males only 36 per cent of the tumours were found in this region ; many more occurred in the connective tissue around the lower abdominal organs.

The masses of tumour tissue frequently adhered to, or invaded at least one of the nearby abdominal organs, *e.g.* liver, spleen, gut, testis, bladder, etc. Thus spread was always by direct growth and infiltration of cells from the main tumour mass.

Occasionally when the ascitic fluid was very cellular multiple implants of tumour growth studded the peritoneal surfaces of all the abdominal organs.

No metastases were found in the lungs.

Apart from the presence of tumours there were no other macroscopic changes, although crystals of benzpyrene, identified by their fluorescence, were frequently present, sometimes embedded on the surface of the liver and spleen.

Histology

Tumours.—The tumours are all sarcomata. They vary in type from a fibrosarcoma to a rapidly growing spindle-cell sarcoma, but many are mixed. The tumour cells are very pleomorphic, and mitoses are very numerous (Fig. 1).

Despite the large size of the tumours there are very few areas of necrosis. No blood vessels are evident within them, except where the tumours have invaded organs with fully formed vessels of their own.

There is no sign of any inflammatory reaction where these tumours invade other organs, though in a few cases the invading tumour is preceded by a small area of necrosis. However, unassociated with the tumours there is much inflammatory reaction, followed by fibrous thickening, at the capsule of the spleen and liver, and more rarely at the kidney capsule.

Liver.—There is an increase in the number of reticulo-endothelial cells in the liver sinusoids, and dividing cells have been seen amongst them. Large numbers of mononuclear cells aggregate in foci in the sinusoids and in the portal tracts. Occasionally giant cells and megakaryocytes are also present in the liver sinusoids, especially where tumours surround, or are invading the liver capsule.

Apart from a little mild centrilobular fatty change the liver cells remain unaltered cytologically.

Spleen.—Many of the tumours arise in the mesentery attached to the spleen. In consequence sheets of tumour cells adhere to the splenic capsule and sometimes penetrate into, and replace much of the normal tissue.

Within the spleen substance there is some hyperplasia of the lymphoid tissue, in which the Malpighian bodies remain approximately normal in size, but the sinusoids fill with lymphocytes. Megakaryocytes are usually more numerous, especially along the edge of an invading tumour, or beneath the capsule if tumour tissue is adherent to it.

The duodenum, kidney, adrenal, testis, and uterus, appear unaffected by the treatment.

A prominent feature of the experiment is the absence of corpora lutea from the ovaries of all but one of the 40 female mice injected with benzpyrene. Even the Graafian follicles appear degenerate in many of the animals killed towards the end of the experiment.

After repeated weekly injections of 0·5 mg. of benzpyrene to 40 female mice the percentage of animals in which tumours developed was only slightly higher, *i.e.* 86 per cent than if a single injection was administered. However the latent period was reduced from 33 weeks to only 18 weeks, *i.e.*, to nearly half. The tumours were similar histologically to those described above, and the histology of the other organs is also as previously described.

(b) *Rats*

Thirty male rats, aged 6 months, were each injected intraperitoneally with 4 mg. of 3 : 4-benzpyrene, contained in 4 c.c. *aq. dest.* Ten female rats, of the same age, each received an intraperitoneal injection of 10 mg. of benzpyrene. The animals were killed when they became ill, or 9 months to one year after the injection.

Only one abdominal fibrosarcoma was found. This was in a male rat and was a large mass which involved the mesentery around the spleen and pancreas, and histologically resembles the fibrosarcomata that developed in mice injected with benzpyrene. However other tumours developed in organs distant from the " site " of injection. Thus, after a single injection of 3 : 4-benzpyrene, 2 female rats developed mammary adenocarcinomata and 2 developed uterine adenocarcinomata, 3 males developed interstitial cell adenomata, and hyperplasia of the adrenal medulla (often extensive enough to be classed as a phaeochromocytoma) occurred in nearly all the rats of both sexes.

Histology

Liver.—There is fibrous thickening of the liver capsule in all the male rats, but the thickening is usually mild and of fairly long standing. In some animals it is gross, but never shows any sign of becoming malignant.

The liver cells remain unaltered cytologically, except in those livers where there is fatty change. This is centrilobular, and is usually very mild, but in a few of the female rats it is more severe, and there are also a few areas of liver cell necrosis. These are responsible for white patches sometimes found on the liver surface at post mortem.

In all the livers the centrilobular veins and adjacent sinusoids are very dilated, and may form wide channels which are packed with red blood cells.

Kidney.—In the kidney there is evidence of some kind of toxic damage to the glomeruli and tubules, though the severity of the damage varies. The glomerular capsule may be distended with protein-containing fluid, and the tufts may be shrunken and partly necrotic, enlarged, lobulated and cellular, or fibrosed. Some of the kidney tubules are dilated and cystic containing eosinophilic protein cast material, while others are shrunken and atrophic and in process of replacement by chronic inflammatory granulation tissue. The blood vessels of the kidney

are often thick-walled and dilated, and many red blood cells pack the spaces between the kidney tubules.

Spleen.—There is no reaction at the splenic capsule. Excessive amounts of haemosiderin are present in the cytoplasm of macrophages, and in some organs the red pulp is very congested.

Adrenal.—In both the male and female rats there are severe changes in the adrenal glands. They are often larger than normal, sometimes unilaterally, so that one gland is four to five times as large as its counterpart.

The cortex is usually very much narrower than normal, chiefly because the zona fasciculata is reduced in width. Frequently the cells of this zone are very vacuolated (Fig. 3), and in the female rats groups of cells may break down leaving a space which fills with red blood cells. The zona reticularis remains quite wide and seems to be packed with cells, even to the exclusion of its large sinusoids which are a constant feature of control glands. The reverse occurs in the zona fasciculata where rows of cells become separated by large lakes of blood.

The main change occurs in the medulla. In nearly all the organs examined this is either replaced by, or contains different sized groups of closely packed cells (Fig. 4, 5, and 6). In some of the smaller nodules the cells are basophilic, but in the larger groups the cytoplasm stains reddish-yellow and is homogenous. The nuclei are large, with thin, deeply-stained nuclear membranes, clear nucleoplasm, and a large eosinophilic nucleolus. Mitoses frequently occur, though none have been found in cells outside the nodules.

The nodules vary in size. Sometimes they are lobed with finger-like processes projecting into the sinusoids. Many are surrounded by a thin endothelium which prevents them from being in direct contact with the adjacent circulating blood. Other nodules are surrounded by cells of the zona reticularis and medulla which are orientated to form a 3–4 layered capsule. The sinusoids of the medulla become grossly dilated so that the nodules are widely separated.

In the larger glands the hyperplastic cells are large and pleomorphic, and are split apart into groups by the dilated sinusoids. Many cells undergo necrosis (Fig. 6) and the nodule may be covered by layers of laminated thrombus. In many cases the appearance of the groups of medullary cells suggests a tumour, such as a phaeochromocytoma. Indeed small nodules of them have been found in the blood vessels within the adrenals, and in one case along the wall of a vessel supplying the gland, but no metastases have been found in any other organ.

Ovary.—Large cysts are present in the ovaries of several female rats. These cysts are of two types: those lined by 1–3 layers of fibrous tissue, and those in which granulation tissue surrounds a central mass of eosinophilic debris, made up of intact and degenerating polymorphs, There are very few normal follicles, although small atretic ones may be present. Many of the large corpora lutea found in these glands contain vacuolated cells, or are breaking down.

Uterus.—At post mortem the uteri of 8 of the female rats were very large, and many contained solid masses. Histologically these were found to consist of either necrotic or calcified debris, or of highly vascular connective tissue polyps which project into the uterine lumen and are attached to its wall by only a narrow stalk, There is hyperplasia of the endometrium in all the uteri and this may be be accompanied by inflammatory reaction (Fig. 7, 8 and 9). The hyperplasia may consist of large papillary processes which project into the lumen, or of cystic dilatations of the uterine glands. In 2 rats the hyperplasia is so great, and the

epithelial cells so actively growing that the condition resembles an adenocarcinoma. Where there is inflammatory reaction the lining epithelium may be eroded away and replaced by granulation tissue, so that the uterine lumen becomes converted into an abscess cavity, and where the inflammation is of long standing large tracts of the uterine wall are fibrosed.

Testis.—Macroscopically the testes of most of the male rats were small, flaccid and blue in colour and the superficial vessels were very obvious. In 3 animals killed at 11 or 12 months after the injection the testes consisted of white, hard, nodular masses enclosed in a thin transparent capsule. These were found to contain large interstitial cell tumours when examined histologically. The seminal vesicles of all the rats were very small.

Histological examination reveals an almost complete absence of interstitial cells between the seminiferous tubules (Fig. 10) except in the 3 animals where there are interstitial cell tumours (Fig. 2). These are nodular and multifocal. Each nodule contains at least 3 types of cells :

(*a*) Large cells, which are few in number and have clear foamy cytoplasm and small, often crenated nuclei.

(*b*) Smaller cells with very slightly vacuolated cytoplasm and a clear well-staining nucleus. These form the bulk of the nodule.

(*c*) Orientated cells, which occur around the periphery of the tumour nodules. These are smaller, basophilic cells, with only a little cytoplasm and a small darkly-staining nucleus. Among these peripheral cells are orientated fibroblasts. There is very little supporting tissue within the nodules and blood vessels are not well developed, so that haemorrhages frequently occur into the tumour. Outside the tumour other interstitial cells are completely absent. No metastases to other organs have been found.

In many testes the seminiferous tubules show some atrophy, which is often patchy, being complete only in the 3 animals having interstitial cell tumours. In some tubules only spermatids and spermatozoa are missing, but in many only Sertoli cells and a few spermatocytes remain. Tubule atrophy seems to develop later than the interstitial cell changes.

The number and size of the blood vessels within the testis appears to increase, so that the organ appears congested. This may be a purely relative effect due to the atrophy of the rest of the testis. The walls of the arterioles are very thick and sometimes hyaline.

Thus it appears that the injection of 3 : 4-benzpyrene as an aqueous colloidal suspension into the peritoneal cavity of mice results in the appearance of abdominal fibrosarcomata in 73 or more per cent of the injected animals, whereas only one such tumour developed in a group of rats similarly treated. However in this species changes occurred in many of the endocrine glands and associated organs, which in some cases resulted in tumour formation at these sites. In mice no changes occurred in any of the endocrine glands or associated organs, apart from the lack of corpora lutea in the ovary.

DISCUSSION

The occurrence of intra-abdominal fibrosarcomata in over 73 per cent of the mice injected intraperitoneally with an aqueous colloid of benzpyrene is similar to that found by Nakehara and Fujiwara (1937), though in this case the benzpyrene

was injected in olive oil. Reference to the literature indicates that very few long-term experiments have been carried out with rats which have received intraperitoneal benzpyrene. Benzpyrene has been injected in lard (Maisin and Coolen, 1934), in collodion sacs (Brock, Druckrey and Hamperl, 1938), and in cholesterol or lard with powdered glass (Des Ligneris, 1940). Varying numbers of local sarcomata were induced.

In the present experiment only one sarcoma has been induced by the intraperitoneal injection of benzpyrene colloid into rats. This is in contrast to the very high incidence of such tumours in similarly treated mice.

The tumours that appeared in mice did so at sites close to those at which benzpyrene is deposited after intraperitoneal injection. This, in the females, was in the mesentery associated with the stomach and spleen, but in the males a larger proportion of tumours and deposits of benzpyrene were found in the lower abdominal cavity, frequently in association with the peritoneal attachments of the testis and epididymis. The correlation of sites of tumours with deposition of benzpyrene appears to support the theory that carcinogenesis is a local process. However, though intra-abdominal deposits of benzpyrene were found in rats as long as 6 months after the injection, only one fibrosarcoma appeared in this species.

There was gross inflammatory response at the capsule of the spleen in mice, and many tumours later developed here but in rats there was neither inflammatory reaction at this site, nor any resultant tumour. This appears to suggest that an initial inflammatory reaction may be associated with tumour formation, but though much inflammatory reaction occurred at the liver capsule of rats no tumours developed there.

As so many of the recorded experiments involving the administration of benzpyrene were chiefly concerned with its carcinogenic activity, few workers have commented on its other pathological effects. However Beltrami (1935) found changes in the livers and spleens of mice painted with benzpyrene, anthracene, or benzene, which he considered indicated a general toxic effect. A more recent study by Benkö, Koltay and Gabor (1953) describes the changes in the livers, spleens and kidneys of mice injected with benzpyrene, 1 : 2 : 5 : 6-dibenzanthracene, or oestrogens. In the liver the sinusoids dilated and aggregations of polymorphs appeared. In addition the liver cell nuclei became swollen. After administration of larger doses of benzpyrene there was some liver cell necrosis followed by regeneration, an even greater increase in the number of polymorphs and round cells, and some increase in the size of the bile ducts. He found very few cell divisions. In the present experiments the livers remained unchanged, apart from the appearance of aggregations of polymorphs and round cells in the sinusoids.

Cramer and Horning (1937) found no changes in the endocrine organs of mice painted with benzpyrene, dibenzanthracene, or tar, but Larionow (1940) reported that the changes in the endocrine organs of mice painted with tar or benzpyrene were similar to those occurring in old age. In the experiments described in this paper in which the benzpyrene was injected intraperitoneally into mice the endocrine organs were indistinguishable from those of control animals, apart from the absence of corpora lutea from the ovaries of the females.

In experiments where rats have been injected with benzpyrene other workers have noted changes other than those associated with tumour formation. Haddow and Robinson (1937), and Haddow, Scott and Scott, (1937), who injected benz-

pyrene or dibenzanthracene into the peritoneal cavity of rats noted some suppression of ovulation, reduced spermatogenesis, and lowering of fertility, but found no changes in the liver, spleen, kidney, bone marrow, suprarenal, or thyroid. However these experiments were only of short duration. Brock *et al.* (1938) noted a loss in testis weight and spermatogenic atrophy in the testes of rats which had collodion sacs containing benzpyrene inserted into the abdominal cavity.

In the experiments described in this paper although only one tumour developed in the abdominal cavity at the " site " of injection in rats, 2 of the female rats developed mammary adenocarcinomata. No such tumours appeared in any of the control rats of the same age. It is suggested that some kind of hormonal imbalance is a causative factor in their development. Similar suggestions have been put forward to explain the action of other polycyclic hydrocarbons. For instance the gastric instillation of methylcholanthrene (Shay, Aegerter, Gruenstein and Komarov, 1949) and the painting of rat skin with 9 : 10-dimethyl-1 : 2-benzanthracene (Marchant, Orr and Woodhouse, 1955), caused an increase in the number of mammary tumours in female rats. As early castration of these rats gave a reduced number of tumours after similar treatment Shay *et al.* suggested that the tumours appeared as a result of hormone imbalance.

The changes that were found in the endocrine organs of the injected rats occurred mainly in the adrenal cortex and medulla, the ovary and testis. Changes were also found in the uterus. The changes in these organs will be discussed under separate headings.

(a) *Changes in the adrenal gland*

The cortical changes, which were particularly severe in the female rats appeared to precede, or were at least independent of the changes in the medulla.

Nodules of hyperplastic adrenal medullary cells are found in nearly all the glands of both male and female rats injected with benzpyrene. In some cases the nodules are extensive enough to be said to form part of a phaeochromocytoma, though no metastases have been found.

Similar changes in the adrenal medulla have been found in untreated rats in old age (Gillman, Gilbert and Spence, 1953 ; Yeakel, 1947 ; and Marine and Baumann, 1945). No changes of this type have been described by other workers who have made post mortem examinations on many thousands of rats of the same age and strain (Curtis, Bullock and Dunning, 1931 ; Saxton, Sperling, Barnes and McCay, 1948 ; and Jayne, 1953), and Gillman suggests that differences in diet, climate, or even genetic strain could be responsible for the different findings.

The possibility that a hormonal imbalance could be responsible for the changes in the adrenal medulla is supported by the work of Moon, Simpson, Li and Evans (1950). Phaeochromocytomata developed in the adrenal glands of male rats which had been repeatedly injected with pituitary growth hormone, but they found no evidence of hypertension, and there were no changes in the reproductive organs.

In the present experiments the changes in the adrenal glands are assumed to have arisen as a result of the injection of benzpyrene, as no such changes occurred in any control rats of a same age and strain. Changes in the testis and associated organs, and in the ovary and uterus, of the treated rats suggest that some hormone imbalance is present, but whether benzpyrene acts via the pituitary gland, as implied by the work of Moon *et al.* is not known.

(*b*) *Changes in the ovary*

Cystic changes occurred in the ovaries of many of the female rats injected with benzpyrene. Similar changes were noted by Rindone (1947) in the ovaries of animals injected subcutaneously with this compound, but Haddow and Robinson (1937) and Haddow, Scott and Scott, (1937), who injected it intraperitoneally into rats only noted a suppression of ovulation. Noble (1938 and 1939) found many corpora lutea in otherwise atrophic ovarian tissue after subcutaneous injection into rats of several oestrogens and polycyclic hydrocarbons.

(c) *Uterus*

Hyperplasia of the uterine endometrium sometimes accompanied by inflammatory reaction occurred in the uteri of all the female rats. In two of these the hyperplasia was so active that the condition resembled uterine adenocarcinoma. Zondek (1937) found similar changes in the uteri of rats which had received continued large doses of follicle hormone. However, the results varied in individual animals. Noble (1938) found pyometra, with uterine hypertrophy in rats injected subcutaneously with oestrogens. In some of these the inflammatory changes spread to the ovaries which contained areas of small cysts. These findings resemble the changes described in the present study.

(d) *Testis*

There were no normal interstitial cells in the testes of the rats injected with benzpyrene, except in the 3 animals with interstitial cell adenomata. There are very few references in the literature to the loss of interstitial cells from the testis

---

EXPLANATION OF PLATES

All sections were stained with haematoxylin and eosin.

FIG. 1.—Section of a fibrosarcoma from a ST. A. male mouse killed 6 months after an intraperitoneal injection of benzpyrene. Many mitotic figures are present in the elongated tumour cells. × 500.

FIG. 2.—Interstitial cell adenoma in the testis of a rat injected with benzpyrene 11 months previously. The tumour is nodular. Completely atrophic seminiferous tubules are present at the top of the photograph. × 200.

FIG. 3.—Vacuolated cortical cells in the adrenal gland of a female rat injected with benzpyrene 7 months previously. Some cells are degenerating. × 335.

FIG. 4.—Adrenal medulla of a male rat injected with benzpyrene 8 months previously. Nodules of hyperplastic cells are separated by large sinusoids packed with red blood cells. × 165.

FIG. 5.—Higher magnification of a nodule of hyperplastic medullary cells. Finger-like outgrowths project into the surrounding sinusoids. × 420.

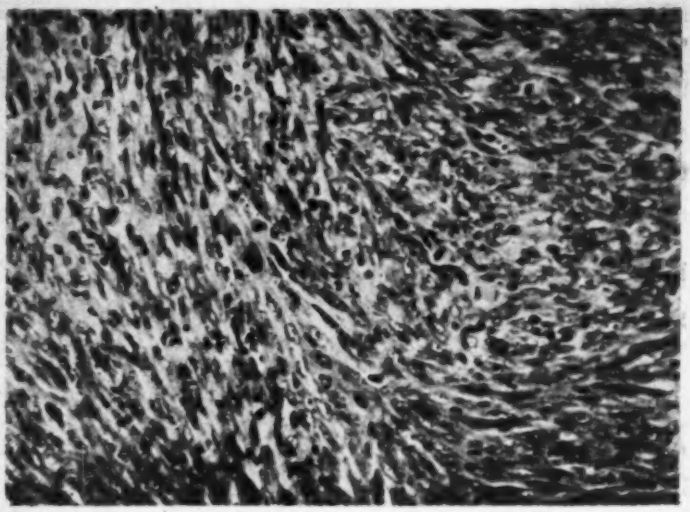
FIG. 6.—Nodule of hyperplastic medullary cells undergoing necrosis. × 420.

FIG. 7.—Endometrial hyperplasia in the uterus of a rat injected with benzpyrene 6 months previously. Pus is present in the uterine lumen, and there is an area of infiltrating polymorphs in the endometrial tissue. × 200.

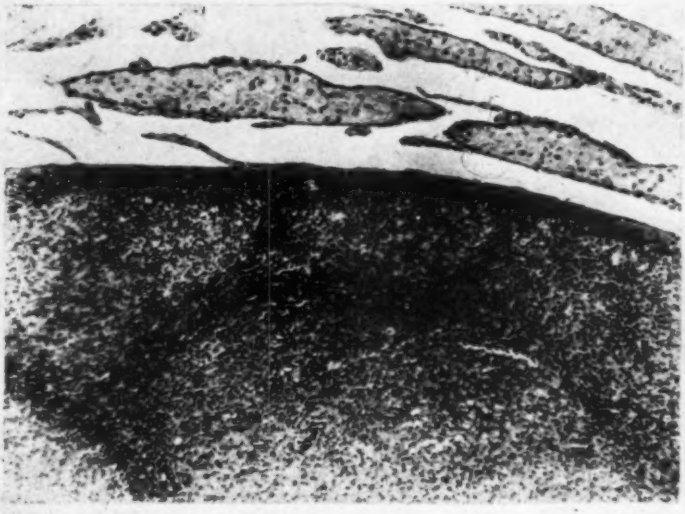
FIG. 8.—Uterus of a rat injected with benzpyrene 6 months previously. The lining epithelium is eroded away and the uterine lumen is filled with mucopus. × 400.

FIG. 9.—Two well-formed abscesses in the uterine wall of a rat killed 9 months after an injection of benzpyrene. × 200.

FIG. 10.—Testis of a rat injected with benzpyrene 8 months previously. There are no interstitial cells, but an increased number of blood vessels are present between the tubules, in which spermatogenesis continues normally. × 200.

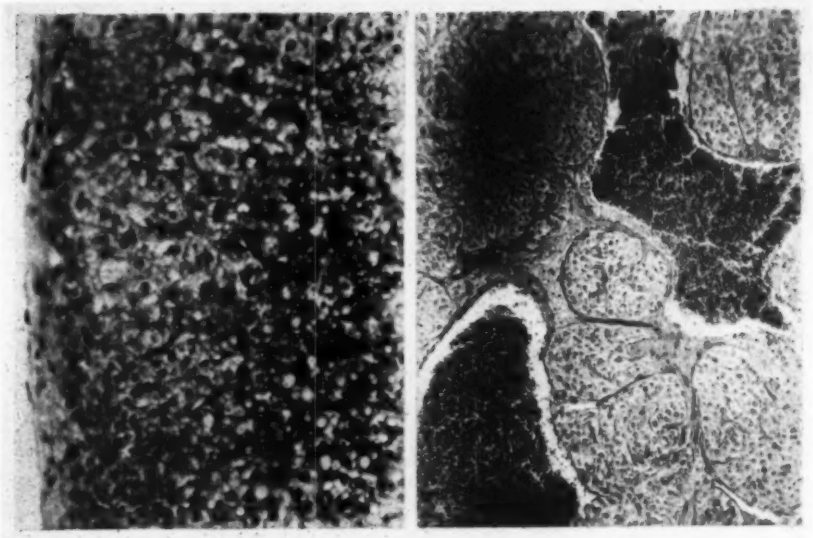


1

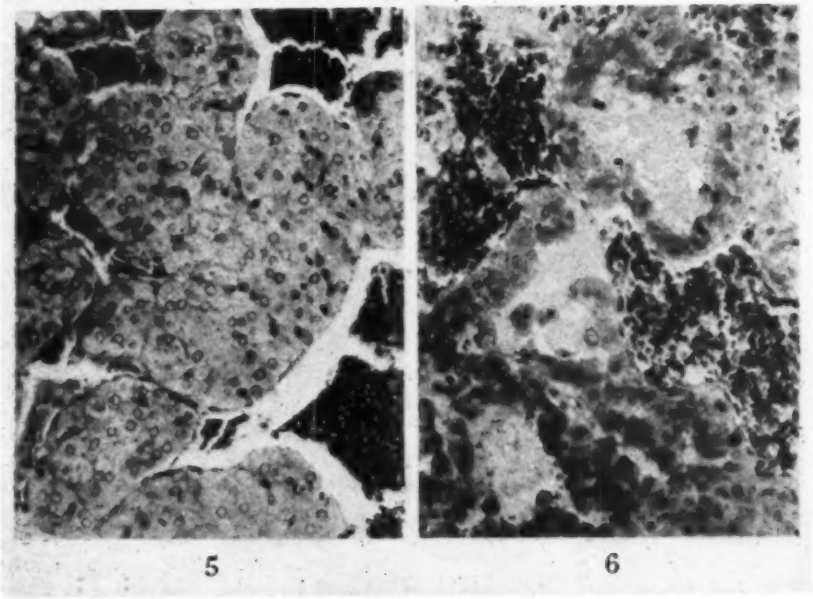


2

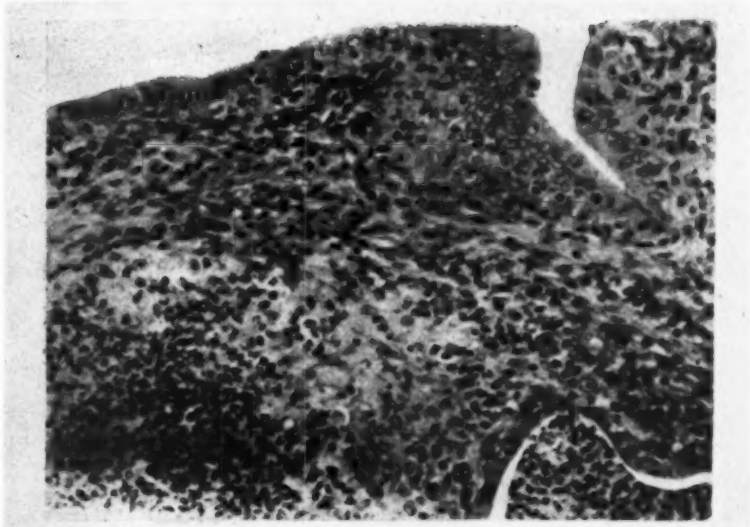
Payne.



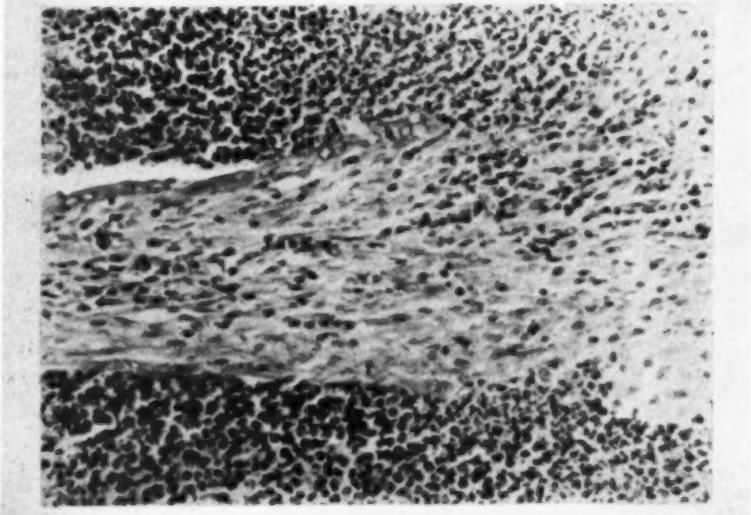
3 4



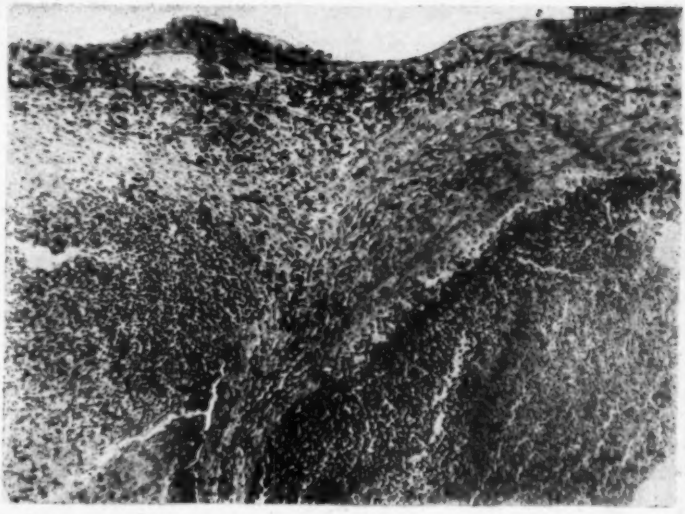
5 6



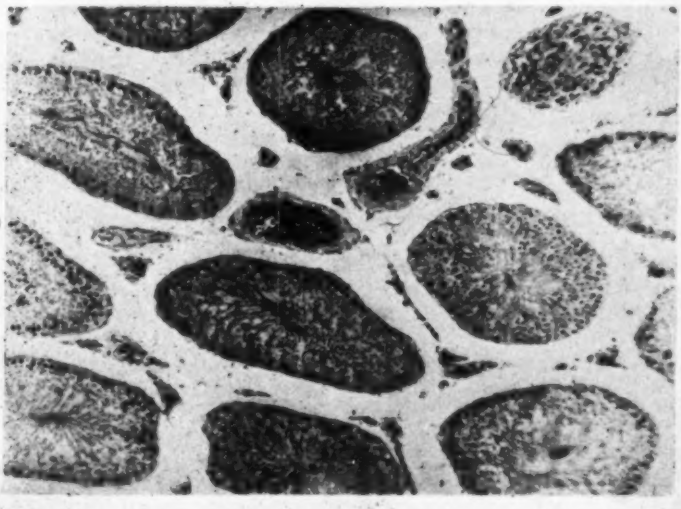
7



8



9



10

but Baker, Schairer, Ingle and Li (1950) found atrophy of the Leydig cells in 80 per cent of rats which had received 21 daily injections of 1 mg. of adrenocorticotropin. They believed that this was due to a reduced production of gonadotropin by the pituitary arising from stress and inanition following treatment.

Spontaneous interstitial cell tumours were found in untreated old rats (Gillman *et al.*, 1953), and Moon *et al.* (1950) found one such tumour in a rat aged 21 months which had been given daily injections of pituitary growth hormone. Again Gillman's findings of changes in older rats appear to differ from those of other workers, but as he suggested, diet, climate or environment may be responsible for these differences. As no tumours appeared in any control animals of the present series it must be assumed that the interstitial cell tumours arose as a result of the experimental treatment.

Seminiferous tubule atrophy, which was extensive in the testes of many of the rats in the present experiments was also noted by Brock *et al.* (1938) and Hamperl (1938), in the testes of rats which had had collodion sacs containing benzpyrene inserted into the peritoneal cavity. Patchy tubule atrophy was found by Rindone (1947) in the testes of some rats and mice injected subcutaneously with benzpyrene, and reduced spermatogenesis occurred in rats injected intraperitoneally with benzpyrene or dibenzanthracene (Haddow and Robinson, 1937; and Haddow, Scott and Scott, 1937).

The blood vessel changes found in the testes of these rats may form part of a possible hypertensive action of benzpyrene, or be a result of the adrenal medullary changes.

The mechanisms of the pathological effects described in this paper remain obscure. They may be due to a direct action of benzpyrene, or one of its metabolites, on the organ concerned, or be mediated through one or more endocrine glands. Experiments designed to elucidate these factors are in progress.

SUMMARY

Over 73 per cent of the mice injected intraperitoneally with an aqueous colloidal suspension of benzpyrene developed intra-abdominal fibrosarcomata. Repeated weekly injections of benzpyrene did not increase the tumour incidence, but reduced the latent period from 33 to 18 weeks.

Of 40 rats similarly treated an intra-abdominal fibrosarcoma developed in one male only. In both male and female rats changes occurred in the endocrine glands and associated organs, which in some cases resulted in the formation of tumours.

In female rats these changes include the presence of cysts in the ovary, endometrial hyperplasia of the uterus, and the development of mammary adenocarcinomata. In male rats interstitial (Leydig) cells are completely absent from the testis, except in 3 animals where interstitial cell adenomata are present. Patchy seminiferous tubule atrophy also occurs.

In both male and female rats there is vacuolation of the cells of the adrenal cortex. In addition hyperplasia of the medulla occurs, frequently to such an extent as to suggest the presence of phaeochromocytomata.

It is concluded that mice are susceptible to the local induction of fibrosarcomata by the intraperitoneal injection of benzpyrene. Such tumours were rarely induced in rats, but changes and tumours occurred elsewhere.

REFERENCES

BAKER, B. L., SCHAIRER, M. A., INGLE, D. J. AND LI, C. H.—(1950) *Anat Rec.*, **106**, 345.

BELTRAMI, W.—(1935) *Tumori*, **9**, 537.

BENKÖ, A., KOLTAY, M. AND GABOR, P.—(1953) *Archiv. Geschwulstforschung*, 5/1, 47.

BOYLAND, E.—(1932) *Lancet*, ii, 1108.

BROCK, N., DRUCKREY, H. AND HAMPERL, H.—(1938) *Arch. exp. Path. Pharmak.*, **189**, 709.

CALCUTT, G. AND PAYNE, S.—(1953) *Brit. J. Cancer*, **7**, 279.—(1954*a*) *Ibid.*, **8**, 554.—(1954*b*) *Ibid.*, **8**, 561.—(1954*c*) *Ibid.*, **8**, 710.—(1955) *Ibid.*, **9**, 426.

CRAMER, W. AND HORNING, E. S.—(1937) *J. Path. Bact.*, **44**, 633.

CURTIS, M. R., BULLOCK, F. D. AND DUNNING, W. F.—(1931) *Amer. J. Cancer*, **15**, 67.

GILLMAN, J., GILBERT, C. AND SPENCE, I.—(1953) *Cancer, N.Y.*, **6**, 494.

HADDOW, A. AND ROBINSON, A. M.—(1937) *Proc. roy. Soc.* Ser. B, **122**, 442.

Idem, SCOTT, C. M. AND SCOTT, J. D.—(1937) *Ibid.*, **122**, 477.

HAMPERL, H.—(1938) *Arch. exp. Path. Pharmak.*, **189**, 187.

JAYNE, E. P.—(1953) *Anat. Rec.*, **115**, 459.

LARIONOW, L. TH.—(1940) *Amer. J. Cancer*, **38**, 492.

DES LIGNERIS, M. J. A.—(1940) *Ibid.*, **40**, 1.

MAISIN, J. AND COOLEN, M. L.—(1934) *C.R. Soc. Biol., Paris*, **117**, 109.

MARCHANT, J., ORR, J. W. AND WOODHOUSE, D. L.—(1955) *Rep. Brit. Emp. Cancer Campgn*, p. 235.

MARINE, D. AND BAULMANN, E. L.—(1945) *Amer. J. Physiol.*, **144**, 69.

MOON, H. D., SIMPSON, M. E., LI, C. H. AND EVANS, H. M.—(1950) *Cancer Res.*, **10**, 364.

NAKEHARA, W. AND FUJIWARA, T.—(1937) *Gann*, **31**, 568.

NOBLE, R. L.—(1938) *Lancet*, ii, 192.—(1939) *J. Endocr.*, **1**, 216.

RINDONE, G.—(1947) *Sperimentale*, **98**, 3.

SAXTON, J. A. Jnr., SPERLING, G. A., BARNES, L. L. AND MCCAY, C. M.—(1948) *Acta Un. int. Cancr*, **6**, 423. (Quoted by Gillman *et al.*, *Cancer*, **6**, 494, 1953.)

SHAY, H., AEGERTER, E. A., GRUENSTEIN, M. AND KOMAROV, S. A.—(1949) *J. nat. Cancer Inst.*, **10**, 255.

YEAKEL, E. H.—(1947) *Arch. Path.* (Lab. Med.), **44**, 71.

ZONDEK, B.—(1937) *Amer. J. Obstet. Gynec.*, **33**, 979.

THE ARTERIAL SUPPLY OF BENZPYRENE-INDUCED TUMOURS IN THE RAT

J. L. BRAITHWAITE

From the Chester Beatty Institute, Institute of Cancer Research, the Royal Cancer Hospital, London, S.W.3, and the Department of Anatomy, University of Liverpool

Received for publication January 16, 1958

THE greater part of the literature which exists on the vascularization of experimental tumours is concerned with the development of the circulation concurrent with the growth of the tumour following the implantation of living tumour tissue or cells. Thus Kanno (1934) studied the effects of transplanting rabbit sarcomata on the blood vessels of internal organs in the rabbit, whilst Hasegawa (1934) investigated the blood supply of subcutaneous implants of sarcomatous tissue in the same animal. Futagami (1936) was concerned with the fate of implants of carcinomatous and sarcomatous tissue on the existing vascular patterns in the rat and noted further the effect of irradiation on the newly-formed vessels. Shinkawa (1939) carried out an extensive investigation on fowl sarcomata derived from implanted tumour tissue. These workers employed the injection of radiopaque material to show up the nutrient vessels of the tumour—a method used originally by Sampson (1912) in his investigation of the blood supply of uterine myomata.

This study is concerned with the arterial supply of the fully developed tumour brought about by the implantation of a benzpyrene tablet into the right flank of the rat, and though similar investigations have been carried out previously notably by Lewis (1927), they have been concerned mainly with the vascularization of the more rapidly growing tumours, such as various types of the Walker sarcoma.

The main points of this contribution are to note the patterns of arterial supply to the tumour and to assess the suitability of carrying out ligation of the nutrient vessels.

MATERIAL AND METHODS

The vascular arrangements of the tumour were studied in eight male rats whose weights ranged between 374 and 570 g. The time between implantation of the benzpyrene tablet and the date of sacrifice varied between 10 and 11 months. The pellet was in every instance inserted into the subcutaneous tissues of the right flank ; in the majority the centre of the tumour was nearer to the axilla than the groin.

The animals were killed by an overdose of chloroform and injection was carried out by passing a polythene tubing fitment attached to a size 1 Record needle into the thoracic aorta and injecting radiopaque medium (Micropaque—Damancy

& Company). The amount of mass required varied between 3 and 5 ml. averaging 4 ml. approximately. The quantity required was related to the weight of the animal and was assessed initially by screening and then taking a series of radiographs following the injection of increasing amounts of injection material.

Following injection a radiograph of the animal was taken on Kodaline film. The vessels to the tumour were then dissected under low power magnification and in selected cases the specimens were photographed. In order to build up a comprehensive picture of the arterial arrangements of the tumour the following additional radiographs were taken in the earlier observations :

1. The tumour after skin reflection and dissection of its main vascular pedicles,
2. the tumour after removal, and
3. slices of the tumour, cut in its long axis.

This method enabled the vessels of supply to be traced from their origins to their terminal ramifications in the interstices of the tumour.

Positives of the radiographs facilitated later study.

FINDINGS

1. *Macroscopic features of tumours—general*

The sizes of the tumours varied between 5 × 2·6 cm. and 1·6 × 1·4 cm., and the distance measured from the central points of the tumour to the right coracoid process ranged between 5·1 cm., and more than 11 cm., although five lay between 5·1 cm. and 6·5 cm. The macroscopic appearances varied between a cyst containing a glairy mucinous fluid with a few small warty excrescences in its wall and a hard scirrhous type of growth. The tumours were all encapsulated and scattered haemorrhagic zones within them were a constant feature. In all except two specimens the tumour was firmly adherent to the thoracic wall.

2. *Arterial supply*

The arteries which supplied the tumour arose from vessels normally supplying the area of integument in which the tumour had grown ; any variations in supply were related to size and site of development of the tumour and any random variations in the origins and mode of branching of the vessels themselves. The intrinsic supply of the tumour was related to its internal morphology (i.e. whether predominantly cystic or solid). The vascularization of four typical tumours will be described and illustrated.

*Tumour B*1

Tumour 3·7 × 2·8 cm.—centre 5·7 cm. from coracoid process. The tumour is adherent to the thoracic wall, and is predominantly cystic.

Arteriograms

Fig. 1.—A branch of the subclavian artery, the subscapular trunk coursing caudally is dividing into larger lateral and smaller medial branches between which the tumour is situated. The lateral division breaks up into numerous fine vessels on

the superficial aspect of the tumour and terminates near its lower pole. The medial branch also courses caudally but lies between the tumour and the thoracic wall.

The vascularity is most dense in the " tumour bed " area, particularly in the caudo-medial quadrant. One or two opacities are seen which are situated in the depths of the tumour ; these indicated haemorrhagic zones due to the friability of the nutrient vessels in certain areas.

Fig. 2.—(The skin has been reflected and the tumour dissected from its bed.)

The arteriogram demonstrates contributions to the medial side of the tumour from the lateral cutaneous branches of three intercostal arteries ; these appear to be running into the dense plexus of vessels at the lower pole. A few fine branches derived from the superficial epigastric artery course cranially to the lower pole. An anastomotic channel is present between the lateral division of the subscapular trunk and the sixth intercostal artery.

The dense plexus of fine vessels in the caudo-medial quadrant is pronounced.

Fig. 3.—(The tumour after removal. It has been cut transversely along its cranial border and opened out.)

The density of vessels at the caudal pole (Fig. 1 and 2) corresponds to the supply of the warty excrescences which were situated in that zone, and tortuous intercostal vessels which were divided during removal of the tumour can be seen coursing to the area.

The areas proximal and distal to this central zone consisting only of smooth cyst wall have a comparatively poor blood supply and the fine vessels on its wall are, in contrast, mainly straight channels.

Tumour B3

Tumour 3 × 2·1 cm. in size is smaller than B1 and its central point is situated 5·1 cm. caudal to the coracoid process. It is firmly attached to the thoracic wall.

Arteriograms

The density of vessels is more evenly distributed than in B1 (Fig. 4). The cranial pedicle divides nearer the tumour than in B1. The medial supply is again afforded by the intercostal arteries, though one, the seventh, is much more developed than the vessels immediately proximal and distal to it. It approaches the tumour at about its centre (Fig. 5). The opacities are situated more laterally than in tumour B1. In the sectioned tumour the intrinsic supply is afforded almost entirely by the enlarged branch of the seventh intercostal artery and opacities occur along the course of some of its branches (Fig. 6).

Tumour B4

Tumour.—the largest in the present series 5 × 2·6 cm., with its central point 7 cm. caudal to the right coracoid process.

Arteriograms

Fig. 7 shows similar vascular pedicles to those described previously—(i) a cranial subscapular supply, (ii) a medial contribution from the lateral cutaneous

branches of the intercostal arteries, although five of these segmental vessels are here concerned in the supply, and (iii) a caudal contribution from the superficial epigastric artery which in this instance is more marked than B1 and B3. The opacities which are concentrated towards the upper surface lie in the depths of the tumour. In the section of the tumour the intercostal branches course transversely to its long axis and give off many fine branches to its parenchyma (Fig. 8).

Tumour B2

Tumour size 3 × 3·4 cm. This was situated close to the groin and at a much more caudal level than the other tumours—it was also firmly anchored to the body wall.

Arteriograms

Fig. 9 shows the following vascular pedicles :

(i) Cranially, contributions from the subscapular artery,
(ii) from the medial aspect from the lateral cutaneous branches of the ninth to the eleventh intercostal and the subcostal arteries.
(iii) Caudally, branches from the epigastric and iliolumbar trunks.

This latter source of supply is more pronounced than in the other specimens studied. Many opacities are present in the caudo-medial quadrant of the tumour. The intercostal contribution to the intrinsic supply of the tumour can be seen in Fig. 10. The intrinsic vessels are tortuous.

DISCUSSION

The arterial supply to the tumours induced by the benzpyrene tablet in the right flank arose from many sources :

(i) Cranially through branches of the subscapular trunk,
(ii) from the medial aspect through the lateral cutaneous branches of one or more intercostal arteries.
(iii) Caudally through the terminal ramifications of the pudo-epigastric trunk, the superficial epigastric and iliolumbar arteries.

An irregular superficial plexus of vessels lay on the surface of the tumour to which all the above named vessels contributed and this communicated with a deeper plexus of vessels within the tumour itself. The main contibution to the latter plexus came from branches of the intercostal arteries.

The findings are in agreement with those of previous authors regarding the sources of supply to the tumour and the fact that haemorrhagic zones (as manifest by leaks of injection material within the substance of the tumour) are a constant feature. The latter are usually most prominent near the centre of the tumour, this being the site farthest removed from the source of arterial blood—they are demonstrated clearly in Fig. 3 and 4 of the paper by Futagami (1936) and also in some of the plates depicting the blood supply to the larger tumours in the series of Shinkawa (1939).

Youngner and Algire (1949) and Algire and Legallais (1951) have produced experimental evidence that reduction of circulation through the capillary bed of

a tumour can be produced by peripheral hypotension and that if this is prolonged tumour damage ensues. This finding would account for the haemorrhagic zones which are such a prominent feature of the larger tumours.

The present work differs in two important respects from that of previous workers —firstly the degree of tortuosity of the nutrient vessels supplying the tumour is not so well developed in the present series, and secondly the stem vessels from which these nutrient arteries arise show no marked evidence of enlargement compared with similar vessels on the contralateral side. The most likely explanation for the absence of these two factors in the present series is the length of time taken for the tumour to grow (in this series, 10–11 months) and it is suggested that during this time the circulatory arrangments have undergone considerable readjustment concurrent with the tumour growth, and this has masked both the collateral channels and the original enlargement of the stem vessels from which the nutrient arteries arose.

Finally, the number of stem vessels from which the tumour derived its blood supply was so great that complete obliteration of these by surgical intervention would be a difficult procedure, coupled with this fact the tumour was firmly attached to the thoracic wall in the majority of cases, making the alernative use of a clamp unsatisfactory for vessel occlusion without damaging the tumour itself.

SUMMARY AND CONCLUSIONS

1. The arterial supply of the benzpyrene tumour occurring in the right flank has been studied in eight male rats.

2. The blood supply of the tumour arose from vessels normally supplying the area of integument in which the tumour had grown—the important ones were :

(*a*) The subscapular trunk from above,

(*b*) terminal ramifications of the superficial epigastric, pudo-epigastric trunk and iliolumbar arteries from below, and

(*c*) the lateral cutaneous branches of intercostal arteries from the medial side.

3. The branches of these vessels contributed to a superficial plexus lying on the capsule of the tumour and a deeper plexus in the interstices of the tumour—there was a rich communication between the two.

4. Differences between findings in the present series and those of previous workers are discussed.

5. In view of the multiple sources of supply and adherence of the tumour to the chest wall obliteration of the arterial supply without causing direct trauma to the tumour was impractical.

I am much indebted to Professor A. Haddow for allowing me all facilities to carry out this work ; also to Professor E. S. Horning for his valuable comments on the manuscript.

The investigation has been supported by grants to the Chester Beatty Research Institute from the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Services.

REFERENCES

ALGIRE, G. H. AND LEGALLAIS, F. Y.—(1951) *J. nat. Cancer Inst.*, **12**, 399.
FUTAGAMI, K.—(1936) *Gann*, **30**, 233.
HASEGAWA, K.—(1934) *Ibid.*, **28**, 32.
KANNO, M.—(1934) *Ibid.*, **28**, 351.
LEWIS, W. H.—(1927) *Johns Hopk. Hosp. Bull.*, **41**, 156.
SAMPSON, J. A.—(1912) *Surg. Gynec. Obstet.*, **14**, 215.
SHINKAWA, T.—(1939) *Nagoya J. med. Sci.*, **13**, 263.
YOUNGNER, J. S. AND ALGIRE, G. H.—(1949) *J. nat. Cancer, Inst.*, **10**, 565.

EXPLANATION OF PLATES.

FIG. 1.—Tumour B1. Vascular pedicles prior to dissection.

FIG. 2.—Tumour B1. Vascular pedicles after skin reflection and partial dissection.

FIG. 3.—Tumour B1. The tumour has been cut along its upper border and opened out.

FIG. 4.—Tumour B3. Vascular pedicles prior to dissection.

FIG. 5.—Tumour B3. Vascular pedicles after skin reflectionand partial dissection (lateral view).

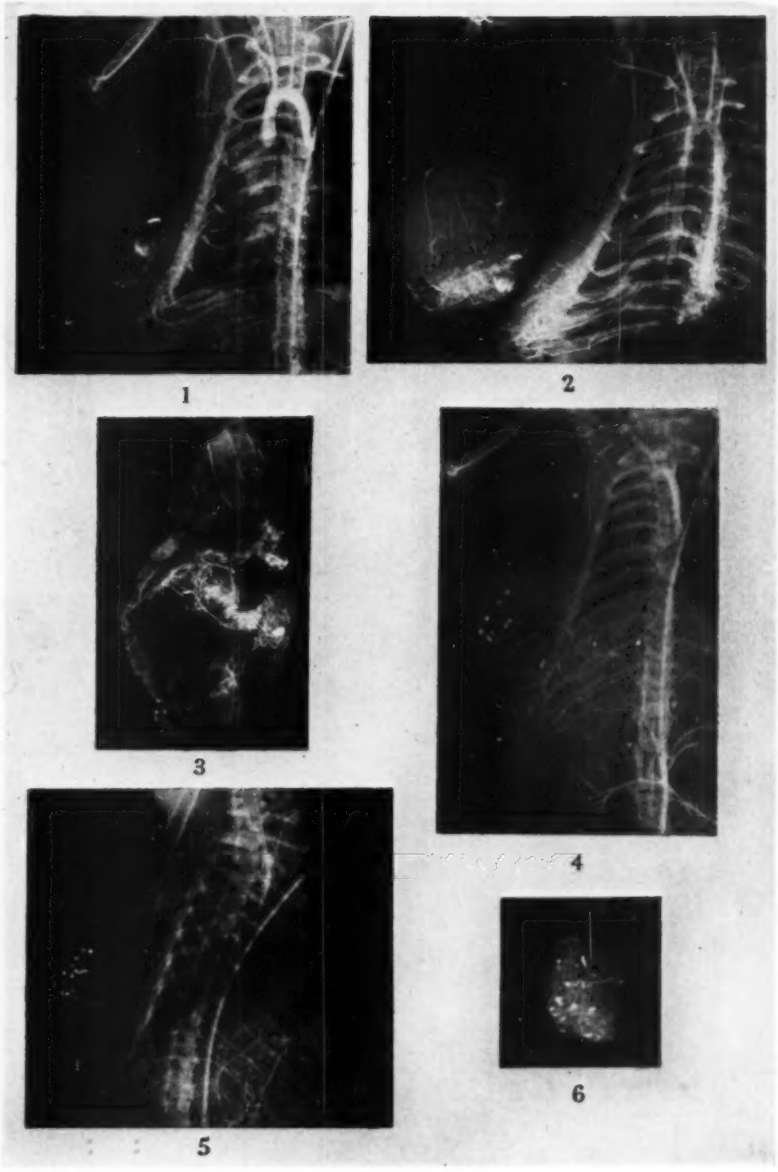
FIG. 6.—Longitudinal section of tumour B3.

FIG. 7.—Tumour B4. Vascular pedicles prior to dissection.

FIG. 8.—Longitudinal section of tumour B4.

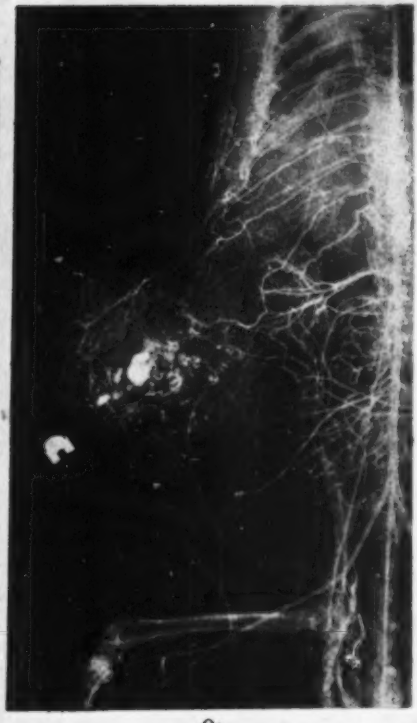
FIG. 9.—Tumour B2. Vascular pedicles prior to dissection.

FIG. 10.—Longitudinal section of tumour B2.



Braithwaite.

7



9

8

10

DISORDERS OF THE BLOOD IN MICE BEARING SPONTANEOUS OR TRANSPLANTED TUMOURS

WILHELMINA J. P. R. VAN EBBENHORST TENGBERGEN
AND O. MÜHLBOCK

From the Laboratory of the Antoni van Leeuwenhoekhuis, The Netherlands' Cancer Institute, Amsterdam, Holland

Received for publication November 29, 1957

DISORDERS of the blood in mice bearing tumours have repeatedly been described. The first disorder to draw attention was leucocytosis; Pappenheim (1911) stated that leucocyte counts can reach very high values even in mice bearing small tumours. The first reports on increased blood volume in rats bearing sarcomata date back to about the same period (Gussio, 1911). It was not until 1946, however, that Furth and Sobel (1946) and other investigators, presented detailed reports on increased blood volume in mice bearing tumours, particularly ovarian tumours. It is surprising that anaemia in tumour-bearing animals received no special attention, whereas the literature comprises numerous reports on anaemia in cancer patients. Since 1950, polycythaemia has frequently been observed in mice bearing luteomata (Gottschalk and Furth, 1951; Wish, Furth and Storey, 1950; Furth and Moshman, 1951). This paper concerns an investigation into disorders of the blood in mice bearing spontaneous or transplanted tumours of varying origin. The aspects discussed are, in order of sequence: the blood volume in normal mice; increased blood volume, anaemia, polycythaemia and leucocytosis in tumour-bearing mice; the significance of necrosis and tumour size with a view to disorders of the blood; extra-medullary haemopoiesis; splenomegaly and cavitary congestive dilation in the organs.

MATERIAL AND METHODS

The mouse strains used were DBA, C57 Bl, O20, C3H, A and CBA and the F_1 hybrids C57 Bl × DBAf (=BDf), O20 × DBAf (= ODf), and Af × DBAf (= Af Df). The 76 tumours used in the investigation can be divided into 12 types, viz.: mammary carcinomata, interstitial-cell testicular tumours, adrenocortical tumours, hepatomata, gastric squamous-cell carcinomata, carcinomata of the jaw, a mixed tumour of the salivary gland, pulmonary papillomata, uterine sarcomata, fibrosarcomata, myxosarcomata and haemangiomata. In view of their transplantability all these tumours, including those showing no infiltration or metastasis, are regarded as more or less malignant in mice. Method of tumour transplantation: subcutaneous, in exceptional cases intraperitoneal, intramuscular or into the tail using a suspension of finely-cut tumour tissue in a physiological saline solution.

Comparisons between the blood volume in normal and that in tumour-bearing animals were made by the decapitation method. Furth and Moshman (1951) state that exsanguination even without transfusion gives useful information. This simple method was used. In consequence only the blood from the large

vessels was measured. Decapitation was done under light ether anaesthesia, and the blood flowing from the trunk and from the head was collected in a small, calibrated centrifuge tube containing 0·5 ml. citrate. The blood volume was read, and the tubes were then centrifuged in order to determine the plasma-cell ratio. The cell counts of the blood were made by the usual methods. The haemoglobin content was determined in a gauged Sahli haemometer. The blood was obtained from the tail without pre-warming.

For morphological examination tumours and organs were fixed in Heidenhain's "Susa" mixture. Sections were stained by haematoxylin-azophloxin staining, and blood and organic smears by Giemsa staining.

The Blood Volume in Normal Mice

In 1840 untreated mice belonging to the same pure and F_1-hybrid strains as the tumour mice under investigation, the blood volume from the large vessels varied from 2·2 to 6·0 ml./100 g. body weight. In animals weighing ± 25 g. the average volume was 4·16 per cent. The large vessels, therefore, probably contain less than 50 per cent of the total blood volume. The quantity of blood proved to be largely dependent on strain and body weight. In the majority of strains, moreover, a difference was found between the sexes: the females as a rule had more blood than the males (Table I).

TABLE I.—*Blood Volume in Untreated Female and Male Mice*

Strains and hybrids	Body weight 20–25 g.				Body weight 25–30 g.			
	Number of mice	♀ ml. average	Number	♂ ml. average	Number of mice	♀ ml. average	Number	♂ ml. average
DBA	40	1·08	59	0·98 S	16	1·33	20	1·23 S
C57 Bl	6	0·95	20	0·84 S	27	1·19	19	0·94 S
O20	53	0·98	70	0·75 S	59	1·09	21	0·99 S
C3H	42	1·06	34	1·00	26	1·19	28	1·16
CBA	15	0·97	6	0·97	37	1·10	27	1·07
F_1BDf	34	1·00	62	0·85 S	10	1·12	60	1·02
F_1ODf	59	0·98	46	0·84 S	20	1·09	44	0·99 S

S = Difference between males and females is significant.

The age of the animals had no demonstrable effect on the blood volume, with the exception of the males of F_1 DBf, in which the blood volume determined later in life was relatively larger than that found in the young animals. Castration, oestrone treatment and presence of the mammary tumour agent failed to cause a change in blood volume. All strains showed a gradual increase in blood volume with an increase in body weight (Table I), but, if expressed in percentage of body weight, there was a relative decrease in the blood volume (Fig. 1).

Burke (1954) likewise found a percentual decrease in blood volume with an increase in body weight in man, opossum, rabbit, rat, sheep, horse, guinea-pig, hog and dog. Courtice (1943), who calculated the blood volume in rabbit, dog, goat and horse, found a similar relationship between blood volume and mass of tissue, chiefly muscle tissue.

The plasma-cell ratio was determined in 690 normal mice. The average haematocrit values in the various strains varied from 33·9 to 42·9 averaging

38·8. According to Wish, Furth and Storey (1950), the total blood volume in mice has a haematocrit value which is 0·88 times that of the venous blood alone. The haematocrit value of 38·8 found in the blood from the large vessels, therefore, would correspond with 44 in the venous blood, i.e. about as much as the value found by Russell, Neufeld and Higgins (1951) in 18 different mouse strains.

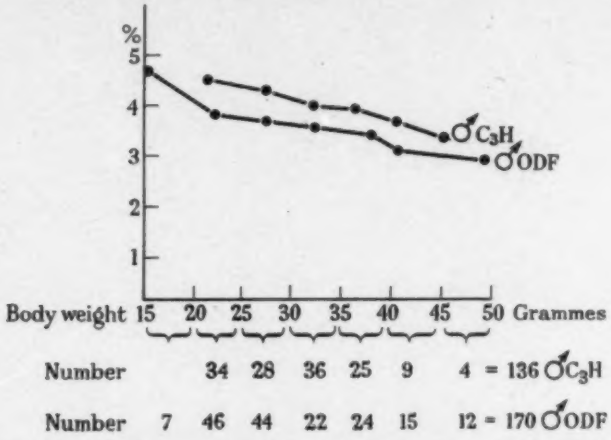


FIG. 1.—Blood volume in untreated mice. Percentage of body weight.

The Blood Volume in Tumour-bearing Mice

Increased blood volume in mice bearing spontaneous and transplanted tumours has been repeatedly described in animals bearing ovarian tumours, (Furth and Sobel, 1946; Furth, 1946; Sobel and Furth, 1948; Bali and Furth, 1949; Cliffton and Wolstenholme, 1949; Furth and Moshman 1951; Mühlbock, 1952; van Nie, 1957) a transplanted testicular tumour (Wolstenholme and Gardner, 1950) and spontaneous mammary tumours (Furth and Moshman, 1951).

In our investigations blood volume determinations were made in a large number of mice bearing numerous different tumours. Many of these animals had a larger blood volume than untreated mice. According to Furth, Beale, Wish and Knoohuizen (1951), the tumour tissue in mice is relatively oligaemic as compared with the organic tissues. A mouse which has gained body weight due to a tumour, therefore, can be compared with a mouse which has gained weight as a result of normal growth (in which case the blood volume per 100 g. body weight is likewise decreased). The blood volume of tumour-bearing mice can therefore be compared with that of normal mice of the same strain, with the same body weight and of the same sex. The haematocrit value shows that the increased blood volume chiefly resulted from an excess of plasma. In a number of cases the cellular volume was also increased. The volume values for whole blood, plasma and cellular mass in untreated mice were regarded as 100 per cent. Values exceeding 120 per cent were regarded as above normal. Extreme examples of the three types of increased blood volume (plasmatic, polycythaemic and mixed) are presented in Fig. 2.

Seven out of the 76 tumours investigated caused no increased blood plasma volume in 79 mice bearing transplants of these tumours. These seven tumours were: two squamous-cell carcinomata of the stomach, two sarcomatoid carcinomata of the jaw, two fibrosarcomata and one myxosarcoma. The remaining 69 tumours produced increased plasma volume in a number of hosts. The types of tumour and the frequency and the degree of increased plasma volume are presented in Table II.

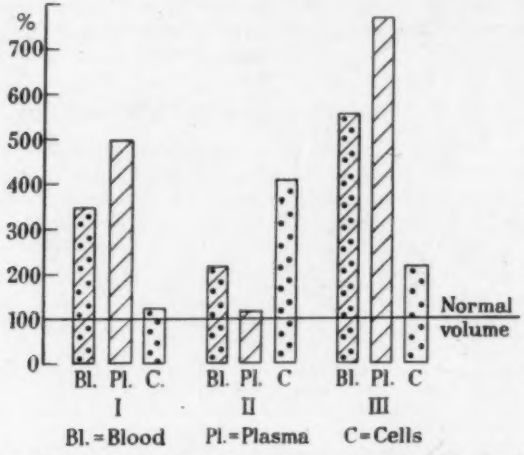


FIG. 2.—Extreme cases of the three forms of increased blood volume.

TABLE II.—*Increased Blood Plasma Volume in Tumour-bearing Mice*

	Number and types of tumours	Strains and hybrids	Plasma volume > 120% of normal value: Number of mice	Plasma volume > 120% of normal value: Average plasma Volume (%)	Plasma volume < 120% of normal value: Number of mice	Plasma volume < 120% of normal value: Average plasma volume (%)
42	Mammary carcinomata	DBA, C57 Bl, O20, C3H, ODf, BDf	612	167 (120–456)	115	103 (61–119)
2	Testicular tumours interstitial-cell type	Af, DB	101	246 (121–739)	5	95 (80–104)
5	Adrenal cortical carcinomata	ODf, AD	100	167 (120–414)	29	103 (80–119)
5	Hepatomata	CBA, ODf, BDf	52	147 (121–193)	57	96 (60–119)
2	Forestomach carcinomata, squamous-cell type	ODf	5	129 (121–137)	16	94 (69–114)
1	Carcinoma of the jaw	O20	4	139 (129–158)	5	99 (83–119)
1	Salivary gland carcinoma, mixed-cell type	BDf	22	220 (143–314)	0	
3	Lung tumours	O20, ODf, BDf	14	136 (121–158)	35	99 (68–119)
4	Uterine sarcomata	Af, ODf	19	156 (125–221)	26	100 (60–119)
1	Spindle-cell sarcoma	ODf	17	160 (121–265)	1	114
1	Myxosarcoma	ODf	5	138 (121–154)	11	103 (80–115)
2	Haemangiomata	ODf	7	136 (121–150)	6	98 (78–117)
69	Tumours	—	958	174 (120–739)	306	99 (60–119)

The highest degree of increased plasma volume was found in the case of the interstitial testicular tumour DB ; the 67 animals bearing this tumour all showed a marked increased plasma volume. Transplantation of this testicular tumour was preceded by splenectomy in 18 mice. These splenectomized mice had a plasma average of 251 per cent ; the 49 mice not splenectomized averaged 252 per cent. In both groups the tumour size was about equal. Splenectomy, therefore, had no effect on the occurrence of increased plasma volume.

Anaemia, Polycythaemia and Leucocytosis

The dilution of the blood by increased plasma volume may produce a pseudo-anaemia, whereas polycythaemia and leucocytosis may be completely or partly masked. For this reason the cell values found in tumour mice with increased plasma volume were converted to the blood volume normal for the mice concerned. These corrected cell values were compared with average normal blood cell values. The normal blood values in the strains used were so variable as to necessitate the use of the over-all average of all strains together as a standard. These average normal blood values were :

Hb . . .	100% = 16·0 g. % (80–127%).
R.B.C. . . .	9·3 million per mm.³ 5·9–13·1 million).
W.B.C. . . .	10,500 per mm.³ 4,500–22,500).
Cell-volume . .	42 μ^3.
Hb E . . .	17 microgamma 12–24 microgamma).
Hb concentration .	0·40 g./c.c. cells.

The term anaemia was only applied to such animals as had haemoglobin values below 75 per cent = 12·0 g./100 ml. The lower limit for polycythaemia was set at 12·5 million erythrocytes/cubic millimetre, and that for leucocytosis at 40,000 leucocytes/cubic millimetre.

A group of 626 mice (31 with a spontaneous mammary-tumour and 595 with transplanted tumours of varying origin) were submitted to determinations including haemoglobin level and red and white cell count in addition to blood volume and haematocrit value. After correction of the blood volume it was found that anaemia occurred in association with all types of tumour, whereas polycythaemia and leucocytosis were found only associated with certain types of tumour (Table III).

Anaemia

Table IV shows the blood cell values for 307 anaemic mice bearing one out of the twelve groups of tumours. All groups showed about the same type of anaemia, viz.:

Haemoglobin content : decreased.
Erythrocyte count : decreased.
Cell volume : normal or enlarged (up to 117 μ^3.) ; seldom below normal.
Hb per cell : slightly decreased.
Hb concentration per cell : as a rule decreased.
Erythrocytes as a rule of unequal size.

The anaemia observed thus resembles that of symptomatic haemolytic anaemia.

TABLE III.—*Anaemia, Polycythaemia and Leucocytosis in Tumour-bearing Mice*

	Number and types of tumours	Strains and hybrids	Number of mice	Computed on normal blood volume: With anaemia (%)	With polycythaemia (%)	With leucocytosis (%)
44	Mammary carcinomata	DBA, C57 Bl, O20, C3H, ODf, BDf	249	60·5	4·0	34·5
2	Testicular tumours, interstitial-cell type	Af, DB	61	29·5	39·3	55·7
5	Adrenal cortical carcinomata	ODf, AD	70	21·4	17·1	0
5	Hepatomata	CBA, ODf, BDf	87	35·6	35·6	0
2	Fore stomach carcinomata, squamous-cell type	ODf	10	60·0	0	80·0
3	Carcinomata of the jaw	O20, ODf	26	50·0	0	38·5
1	Salivary gland carcinoma, mixed-cell type	BDf	13	76·9	0	30·8
3	Lung tumours	O20, ODf, BDf	26	38·5	0	15·4
4	Uterine sarcomata	Af, ODf	36	69·4	0	19·4
3	Spindle-cell sarcomata	C3H, ODf	21	57·1	0	14·3
2	Myxosarcomata	C3H, ODf	19	42·1	0	31·6
2	Haemangiomata	ODf	8	100·0	0	12·5
76	Tumours	—	626	307 mice	77 mice	163 mice

TABLE IV.—*Anaemia in Tumour-bearing Mice*

(computed on normal blood-volume)

	Number and types of tumours	Strains and hybrids	Mice with anaemia	Hb Sahli = g.% average	R.B.C. mm.³ 10^6 average	Cell vol. μ^3 average	HbE micro γ average	Hb conc. g./c.c. cells average
44	Mammary carcinomata	DBA, C57 Bl, O20, C3H, ODf, BDf	151	56=9·0	5·27	50·8	14·4	0·30
2	Testicular tumours, interstitial-cell type	Af, DB	18	45=7·2	6·39	53·6	11·5	0·23
4	Adrenal cortical carcinomata	ODf, AD	15	52=8·3	5·23	56·5	16·1	0·29
5	Hepatomata	CBA, ODf, BDf	31	51=8·1	5·52	48·3	15·3	0·33
2	Fore stomach carcinomata, squamous-cell type	ODf	6	66=10·6	7·68	42·6	13·8	0·31
3	Carcinomata of the jaw	O20, ODf	13	54=8·8	5·34	41·2	16·7	0·42
1	Salivary gland carcinoma, mixed-cell type	BDf	10	44=7·0	5·20	44·8	14·0	0·31
3	Lung tumours	O20, ODf, BDf	10	60=9·6	6·71	34·9	14·7	0·45
3	Uterine sarcomata	ODf	25	50=8·0	5·10	42·1	16·0	0·34
3	Spindle-cell sarcomata	C3Hf, ODf	12	69=11·0	6·81	45·2	16·7	0·38
2	Myxosarcomata	C3Hf, ODf	8	65=10·4	7·51	40·4	14·1	0·36
2	Haemangiomata	ODf	8	23=3·7	2·34	65·0	16·7	0·30
74	Tumours	—	307	52=8·3	5·67	47·2	15·3	0·32
	Normal values general average			100=16·0	9·30	42·0	17·0	0·40

TABLE V.—*Polycythaemia in Tumour-bearing Mice*

(computed on normal blood-volume)

	Number and types of tumours	Strains and hybrids	Mice with polycythaemia	Hb Sahli=g.% average	R.B.C. mm.^{3} 10^6 average	Cell vol. μ^3 average	HbE micro γ average	Hb conc. g./c.c. cells average
7	Mammary carcinomata	C3H, O20, BDf, ODf	10	128=20·5	14·81	36·2	13·8	0·39
2	Testicular tumours, interstitial-cell type	Af, BDf	24	140=22·4	16·07	45·1	13·9	0·32
4	Adrenal cortical carcinomata	ODf, AD	12	111=17·7	13·97	36·5	12·9	0·36
3	Hepatomata . .	CBA, ODf, BDf	31	151=24·1	16·78	41·2	14·5	0·36
16	Tumours . . .	—	77	139=22·3	15·82	40·9	14·0	0·35
	Normal values general average .			100=16·0	9·30	42·0	17·0	0·40

Polycythaemia

Polycythaemia was found in association with only 16 of the 76 different mouse tumours (Table III), viz.: in 7 of the 44 mammary-tumours, both the interstitial testicular tumours, 4 of the 5 adrenocortical tumours, and 3 of the 5 hepatomata. Polycythaemia occurred in 77 of the 215 mice bearing these tumours. In those cases in which the highly cellular blood was not diluted as a result of an increase in plasma, the polycythaemia was accompanied in the live animals by the strikingly red colour of the nozzle and the paws, and by considerable restlessness. The blood values of the polycythaemic mice are presented in Table V. The highest erythrocyte count observed was 31·4 mm.$^3 10^6$ = 3·38 times the normal value. The polycythaemia disappeared within 2 to 3 weeks after extirpation of the tumour The disorder, therefore, is symptomatic polycythaemia or erythrocytosis.

Leucocytosis

Eighty different tumours (growing in 698 mice) were investigated with a view to leucocytosis. Forty-eight of these 80 tumours were found to produce leucocytosis, but not in all animals bearing the tumours. Leucocytosis was not found in any of the 5 adrenocortical tumours nor in any of the 5 hepatomata (investigated in 70 and 87 mice respectively). The average leucocyte counts of the positive and the negative animals are presented in Table VI. The mice listed negative in the second column of Table VI have, however, an average leucocyte count more than twice the normal value. The leucocytosis was invariably of the granulocyte type. Immature cells were frequently found in the blood, but the leucocytosis was not transplantable. Extirpation of the tumour was followed by a decrease in the leucocyte count to normal within about a week; it remained normal thereafter.

Mice bearing the testicular tumour DB invariably showed increased plasma volume. In 14 animals the tumour was extirpated. These animals had a leucocytosis with an uncorrected average count of 41,600 immediately before extirpation of the tumour. Two days after extirpation all animals showed an increase in the leucocyte count per c.mm. to 2 to 3 times the original value (from 41,600 to 97,800 on average). Three to 8 days later normal leucocyte counts were found

TABLE VI.—*Leukaemoid Reaction in Tumour-bearing Mice*

(computed on normal blood-volume)

Number and types of tumours	Total number of mice	Leucocytosis observed							No leucocytosis observed			
					> 40,000 leucocytes		< 40,000 leucocytes					
		Number of tumours	Strains and hybrids		Number of mice	Leucocytes average	Number of mice	Leucocytes average	Number of tumours	Strains and hybrids	Number of mice	Leucocytes average
48 Mammary carcinomata	292	35	DBA, C57 Bl, O20, C3H, ODf, BDf		123	111,400	102	22,400	13	DBA, C3H, C57 Bl, ODf	67	17,900
2 Testicular tumours, interstitial-cell type	90	1	DB		61	164,900	10	22,500	1	AD	19	15,900
5 Adrenal cortical carcinomata	70	0	—		0		0		5	ODf, AD	70	14,900
5 Hepatomata	87	0	—		0		0		5	CBA, ODf, BDf	87	13,000
2 Fore stomach carcinomata, squamous - cell type	10	2	ODf		8	56,100	2	31,700	0	—	0	
3 Carcinomata of the jaw	26	3	O20, ODf		10	57,400	16	25,700	0	—	0	
1 Salivary gland carcinoma, mixed-cell type	13	1	DBf		4	62,900	9	27,900	0	—	0	
3 Lung tumours	26	1	ODf		4	100,800	8	20,000	2	O20, BDf	14	14,400
4 Uterine sarcomata	36	1	ODf		7	71,100	4	17,700	3	Af, ODf	25	14,800
3 Spindle-cell sarcomata	21	2	C3Hf, ODf		3	54,500	13	21,000	1	ODf	5	16,000
2 Myxosarcomata	19	1	ODf		6	48,600	5	22,200	1	C3Hf	8	7,400
2 Haemangiomata	8	1	ODf		1	43,400	1	14,400	1	ODf	6	13,100
80 Tumours	698	48	—		227	119,400	170	22,900	32	—	301	14,800
		Highest value found : 668,900 Tumour weight 1·8–24·2, average 6·4 g.					2·1–16·0 average 5·0 g.		Normal leucocyte count general average : 10,500 Tumour weight 1·0–32·1, average 5·1 g.			

in the peripheral blood. The initial increase in the leucocyte count was probably a relative increase due to a decrease in plasma volume.

Relapse of a tumour after extirpation was associated with a return of the leucocytosis, which is therefore a leukaemoid reaction (Dunn, 1954).

Frequency of the Four Disorders of the Blood

Although the 76 tumours all caused disorders of the blood, yet not every tumour was capable of producing each of the four above-mentioned disorders and not every mouse, moreover, developed the disorders of the blood which it might develop due to the tumour it was bearing. The mice frequently developed several disorders simultaneously.

Disorders of the Blood Associated with Spontaneous Tumours

The above-mentioned four disorders of the blood were also found in association with spontaneous tumours. A group of 31 mice bearing spontaneous mammary tumours weighing 2–11 g., average 6 g., included 27 with increased plasma volume, 12 with anaemia, 2 with polycythaemia and 3 with leucocytosis. The 31 tumours were further investigated as transplanted tumours (in 1st, 5th, 10th, 15th and 20th passage). The increased plasma volume showed about the same average frequency throughout all passages, as did the polycythaemia. Anaemia and leucocytosis, however, showed a significant increase. Repeated transplantation of tumours, however, need not necessarily give rise to a leukaemoid reaction. The animals bearing adrenocortical tumours (up to passage 24) and hepatomata (up to passage 62) never developed leucocytosis.

Tumour Mice with Normal Blood

Among the 626 mice there were 64 (10·2 per cent) with all blood values within the established normal limits. The average tumour weight in these animals was 5·0 g., the average weight of tumours which did give rise to disorders of the blood was also 5·0 g. These 64 animals were distributed over all the strains used. The highest percentage of mice with normal blood values was found among the animals bearing fibrosarcomata and myxosarcomata, and the lowest percentage among the animals bearing mammary tumours.

Causative Factors

Necrosis

Leucocytosis has been studied for half a century, not only in animals bearing tumours, but also in human cancer patients ; Hensler (1953) mentions 13 authors who have reported on the subject since 1893. Yet the aetiology is still completely obscure. It is generally presumed that necrosis and ulceration of the tumour should be regarded as factors in the causation of leucocytosis. It is understood, however, that these factors alone offer no adequate explanation of the phenomenon. Necrotic and ulcerating tumours are frequently seen, but leucocytosis is rare in association with tumours in man. Bateman (1951*a*), with cultures of bacteria isolated from mouse tumours associated with leucocytosis, obtained only a slight increase in the leucocyte count ; aureomycin and streptomycin had no effect on the leukaemoid reaction.

Necrosis was frequently seen in the mouse tumours investigated. The tumours causing the highest degree of necrosis were often those with the highest rate of mitosis. The quantity of the necrosis varied from hardly demonstrable to about 90 per cent of the tumour. An attempt was made to control necrosis formation in a few transplanted tumours known to produce considerable necrosis, by altering conditions of tumour growth. The following methods of transplantation were used: (1) multiple subcutaneous implantations with small quantities of suspension; (2) intraperitoneal implantation; (3) intramuscular implantation; (4) implantation into the tail. By none of these methods was it possible to prevent or to reduce necrosis formation. Implantation of exclusively necrotic tumour tissue caused no leucocytosis. The necrotic material was absorbed, except in two mice, in which the transplanted necrosis was sloughed. These two animals showed a rise in the leucocyte count to 35,000 and 36,000, respectively, before penetration of the skin—a rise to below the limit established for tumour leucocytosis—which was rapidly followed by a fall.

Table VI presents a group of tumours which never produced leucocytosis; this group comprised 52 mice showing a highly necrotic tumour; on the other hand 24 animals with very marked leucocytosis (average leucocyte count 170,000) showed no or hardly any necrosis in the tumour. In a large number of mice the degree of necrosis in the tumour was evaluated both macroscopically and microscopically. In this group of animals the disorders of the blood associated with marked necrosis were compared with those in animals bearing a tumour completely or almost completely free of necrosis. Cases of moderate necrosis were not taken into account.

For each disorder of the blood only those mice were compared which bore tumours capable of producing the disorder in question. The values found are given in Table VII.

TABLE VII.—*Comparison of Markedly Necrotic Tumours with Non-necrotic Tumours*

	Mice with markedly necrotic tumours	Mice with non-nectoric tumours
Increased plasma volume	87 on 136 = 64·0% Average plasma vol. = 176%	172 on 216 = 79·6% Average plasma vol. = 188%
Anaemia	77 on 139 = 55·4% Average Hb = 53% = 8·5 g.% Average R.B.C. = 5·47 mm.^{3} 10^6	100 on 224 = 44·6% Average Hb = 51% = 8·2 g.% Average R.B.C. = 5·64 mm.^{3} 10^6
Polycythaemia	5 on 29 = 17·2% Average R.B.C. = 17·81 mm.^{3} 10^6	20 on 71 = 28·2% Average R.B.C. = 16·30 mm.^{3} 10^6
Leucocytosis	45 on 94 = 47·9% Average W.B.C. = 107,800 mm.^{3}	47 on 102 = 46·1% Average W.B.C. = 116,500 mm.^{3}

Increased plasma volume was significantly less frequently associated with markedly necrotic tumours than with non-necrotic tumours, $\chi^2 = 10{\cdot}52$. Anaemia on the contrary occurred significantly more often associated with necrotic tumours, $\chi^2 = 3{\cdot}97$. The frequency of polycythaemia and of leucocytosis was the same in both groups, $\chi^2 = 1{\cdot}31$, respectively 0·06. This shows that necrosis was not the factor causing the disorders, except possibly in the case of anaemia. Nor was necrosis demonstrated to promote the severity of the disorders.

Tumour size

Each of the four disorders (increased plasma volume, anaemia, polycythaemia and leucocytosis) were observed even in animals with very small tumours. The average tumour size and weight spread was about the same in the groups with the various disorders of the blood and the group with normal blood (Table VIII).

TABLE VIII.—*Tumour Weight as Related to Blood Disorders*

	Mice with				
	Increased blood plasma volume	Anaemia	Polycythaemia	Leucocytosis	Normal blood
Tumour weight in g.	0·8–32·1 av. 5·3	1·0–24·2 av. 5·3	0·7–12·5 av. 4·9	1·8–24·2 av. 6·4	1·2–14·0 av. 5·0
Number of mice	958	307	77	227	64

Tumour size is therefore not a factor essential in the aetiology of disorders of the blood. Tumour size is of significance, however, with a view to the degree of increase in plasma and the degree of increase in the leucocyte count. Fig. 3

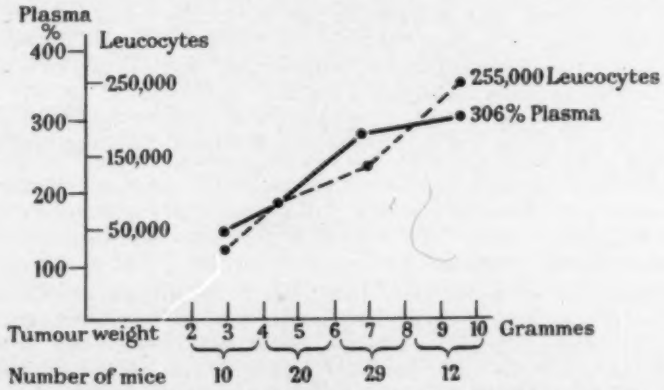


FIG. 3.—Effect of the size of the tumour on the plasma volume and on the leukaemoid reaction (testicular tumour DB).

shows the regular increase in plasma volume and in leucocyte count with an increase in the weight of testicular tumour DB. Increasing tumour weight was not found to be associated with increasing polycythaemia or anaemia. There was no correlation between the duration of tumour growth and the disorders of the blood.

Hormonal factors

If neither the necrosis nor the tumour quantity can be regarded as causative factors, then the quality of the living tumour tissue must be of aetiological importance. As has been pointed out, increased blood volume was often associated with ovarian tumours. Granulosa-cell tumours have been described by van Nie (1957) of our Institute. Ten of 17 tumours with an oestrogenic effect produced

increased blood volume. In men, Bateman (1951*b*) observed a relationship between sex hormones and blood volume in three cancer patients who showed increased blood volume following administration of large doses of stilboestrol; withdrawal of the drug resulted in reversion toward normal values. The variety of the mouse tumours without an oestrogenic effect but capable of producing increased blood volume, as presented in Table II, proves that these hormones cannot be the sole cause of the increase in plasma volume. Sex hormones possibly enhance the effect of another causative factor, which also causes increased blood volume in the case of tumours without an output of hormones. In the aetiology of polycythaemia, too, an influence of sex hormones has been considered. Polycythaemia is found in the case of masculinizing luteoma in mice (Wish, Furth and Storey, 1950; Gottschalk and Furth, 1951). Of the 16 tumours causing polycythaemia described here, however, the same can be said as of the increased blood volume: tumours without a hormonal effect can also give rise to polycythaemia. Tumours possibly stimulate the hypophysis to produce a specific hormone, which is assumed to be connected with erythropoiesis (Querido and Overbeek, 1938; Dyke, Contopoulos, Williams, Simpson, and Lawrence, 1954; Contopoulos, Ellis, Simpson, Lawrence and Evans, 1954). The anaemia could be a result of a direct effect of tumour substances on the blood. Ponder and Nesmith (1952) found that extracts of mouse tumours had a more marked haemolytic effect than extracts of the lungs and kidneys of mice of the same strain.

This investigation has again failed to throw any light on the mechanisms of action of the leucocytosis. A striking feature was found in the fact that the 5 adrenocortical tumours and the 5 hepatomata were all incapable of producing leucocytosis in any of the 157 mice investigated. In no case was marked leucocytopenia observed.

Morphological Investigations

Extramedullary haemopoiesis

Granulopoiesis.—In tumour mice with leucocytosis granulopoiesis was usually observed in the haemopoietic organs, Although there was a variation in the degree of activity in different organs of a single mouse with leucocytosis, in the group as a whole the leucocyte count in the peripheral blood and the average myelopoietic activity in a particular organ, for example the spleen, was closely correlated. The lungs, too, as a rule contained granulocytes (Fig. 4), the average quantity of which likewise corresponded to the leucocyte count of the peripheral blood. It is a question whether the lung has a granulopoietic function of its own or that both the mature and the immature granulocytes have been transported to the lung.

Weisberger, Guyton, Heinle and Storaasli (1951) found that the lungs were much more active in the removal of lymphocytes and granulocytes than other organs. Therefore they regard the lung as a homoiostatic organ which ensures the balance of the white blood cells. Six mice, however, with heavy leucocytosis had none or only a few granulocytes in the lungs. The same uncertainty as regards transportation or local production of granulocytes pertains in the kidney, in which immature and mature granulocytes are also found in the case of marked leucocytosis, especially in the glomeruli. In the liver and adrenals granulopoiesis occurred not so regularly as it did in the spleen, lymph nodes and lungs. Only

in mice bearing the testicular tumour DB granulopoiesis was usually heavy in the liver (Fig. 5).

Twenty-two animals were splenectomized prior to transplantation of the testicular tumour DB. Leucocytosis occurred in 81·1 per cent of these splenectomized animals (average 165,400 ; maximum 429,300 cells), as against 87·8 per cent of 49 intact animals with the same testicular tumour (average 165,100 ; maximum 517,500 cells). The splenectomy therefore did not reduce the degree of leucocytosis. In the splenectomized leucocytotic mice marked to very-marked granulopoiesis was found in 100 per cent of the lymph nodes ; in the intact animals this was 78·4 per cent. The difference is significant ($\chi^2 = 4{\cdot}42$). The other organs showed no difference in the degree of granulopoiesis between the splenectomized and the intact groups. The granulocyte production of the spleen, therefore, would seem to be taken over mainly by the lymph nodes after splenectomy.

Forms of Granulocyte Nuclei in the Haemopoietic Organs

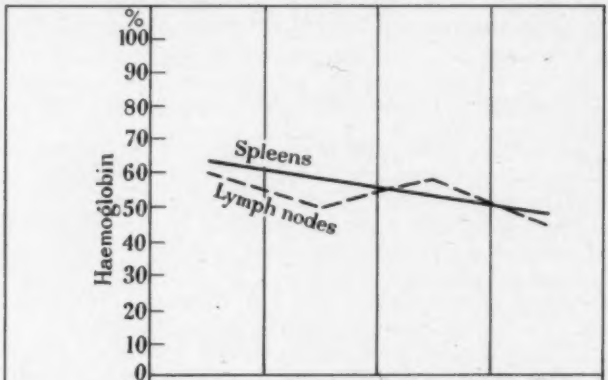
In mice granulocytes arise from myeloblasts, developing via the myelocyte form to metamyelocytes, the nucleus of which initially consists in a thick smooth ring with a small lumen. On maturation of the cell this ring becomes thinner and serrated, the lumen becoming wider meanwhile. In mature cells the ring may remain closed or open up, showing lobulated structures. Petri (1933) found a predominance of closed rings in the peripheral blood of mice ; Barnes and Sisman, (1939) and De Bruyn, Korteweg and Kits van Waveren (1949) found a majority of open forms. In the haemopoietic organs of the tumour mice described here the majority of the granulocytes showed a certain type of nucleus which seemed to be specific to the organ. In the liver and the adrenals the myeloid cells often showed no maturation whatever (Fig. 5). This was particularly so in animals with the interstitial testicular tumour DB. In the case of this tumour, the total mass of myeloid cells in the periportal and capillary regions of the liver may exceed that of the liver cells ; myeloblasts and myelocytes were usually found exclusively, in some cases with a small number of young metamyelocytes. Foci of mature, granulocytes were occasionally seen, but only round the large vessels. After vital staining with trypan blue it was found that such livers contained a normal number of Kupffer cells. The granulocytes of tumour mice showed all stages from myeloblasts to mature cells in the spleen. The majority of the mature granulocytes in the spleen showed a thin annular nucleus without or with inconsiderable serration, i.e. a young maturing form (Fig. 6). In the lymph nodes and in the thymus the mature granulocytes showed more often an open, serrated type of annular nucleus, as well as S-shaped and lobulated nuclei (Fig. 7). Maturation in these lymphoid organs, therefore, reached a higher degree than that in the spleen. The often enormous masses of granulocytes in the alveolar walls and vessels in the lungs, for the most part contained hypersegmented and looped nuclei (Fig.4) The immature forms were small in number and the metamyelocytes likewise showed a deviating form in that serration was often already present in the thick ring. Mitoses were found. The same typical granulocyte nuclei were found in the lungs of a mouse without a tumour, but suffering from leucocytosis resulting from an intestinal abscess. The nuclei of the mature granulocytes in the kidney, too, were hypersegmented. The hypersegmentation possibly denotes the onset

of karyorrhexis of imported granulocytes. The number of loose nuclear particles, however, is but small.

The environment in which the myeloid cells develop would, therefore, seem to influence the degree of maturation of granulocytes before these enter the circulation. This perhaps affords an explanation of the different proportions under which the nuclear types have been observed in the circulation in mice.

Erythropoiesis

In anaemic and polycythaemic mice extramedullary erythropoiesis occurred in the haematopoetic organs (Fig. 8, 9). In the lung too, immature red blood



	No	Slight	Moderate	Extensive
	Erythropoiesis			
No. of spleens	32	59	53	49
No. of l. nodes	29	26	19	23

FIG. 11.—Erythropoiesis as related to degree of anaemia.

cells were found, but seldome more than 4 or 5 in the same alveolar wall. Fig. 11 shows the parallelism between the degree of erythropoiesis in the spleen and the lymph nodes and the degree of anaemia. There was sometimes erythropoiesis in the organs of animals without anaemia but this may well be explained by compensation of the anaemia in these animals by increased erythrocyte production. In 34 polycythaemic mice—all with the testicular tumour DB, with an average of 16·0 million R.B.C. per mm.^{3} in normal blood volume (maximum count 34·1)—the spleen, lymph nodes, liver, lungs and adrenals were tested for erythropoiesis. In 13 of these animals there was no increased erythropoiesis in the organs in spite of the high number of R.B.C. in the peripheral blood (up to 25·5 million). Of the remaining 21 polycythaemic animals, 9 had a fairly marked erythropoiesis which, as a rule, was confined to a single organ. This moderate production cannot explain the marked increase in erythrocytes. It is known that in mice the bone marrow has very little reserve, but that some segments in the tail can be utilized

(Dunn, 1954). These sites were not investigated, so no explanation can be given for the cause of the polycythaemia.

The spleen is regarded as one of the chief breakdown sites for erythrocytes. Splenectomy would consequently be able to promote the polycythaemia. In the group of splenectomized mice, however, the frequency of polycythaemia was as high as in the group of intact mice; the average degree of polycythaemia, too, showed no difference between the two groups. A number of splenectomized mice had anaemia; as has been pointed out, splenectomy did not influence the abnormal blood volume either. The spleen would therefore not seem to have an unusual position among the organs involved in the haematological anomalies discussed, although it participates so actively in haemopoiesis that splenomegaly may result.

Splenomegaly

The spleen in tumour mice is known to be moderately enlarged as a rule. The causes of the splenomegaly in general may be oedema or hyperaemia, amyloidosis or an increase in cells. The weight of normal mouse spleens as a rule varies from 80 to 140 mg., but may be more. The average splenic weight in 472 tumour mice was 271 mg. The enlargment would seem to result mainly from increased haemopoiesis. The average weight of 20 spleens with a considerable production of both erythrocytes and granulocytes was 466 mg. Marked erythropoiesis with normal granulocyte production was found in 76 spleens with an average weight of 320 mg., whereas that of 35 spleens with a marked granulopoiesis and normal erythrocyte production was 224 mg. Thirty-eight spleens did not show abnormal haemopoiesis; the average weight was 164 mg. i.e. hardly more than normal. Increased blood volume did not influence the splenic weight. Splenomegaly due to cavitary dilatations (see later) was rare. The part of an increase in the megakaryocyte count in splenomegaly was not determined; this was impossible because an abnormally high megakaryocyte count was nearly always associated with haemopoietic activity.

Megakaryocytes

The megakaryocytes in the spleen showed a marked increase in 224 of 472 tumour mice. Megakaryocytes were also often found in the liver (up to 70 in a single section of 9 sq. mm.), and exceptionally also in the lungs, lymph nodes and adrenals. There is a difference of opinion as regards the origin of the megakaryocytes in the spleen; they are believed to develop from a basophile lymphoid mother cell, (Klemperer, 1938), or directly from myeloblasts (Ham, 1950). In the latter case an increase in megakaryocytes could be expected to be associated with myelopoiesis in the spleen of tumour mice. In 81 of the 224 spleens rich in megakaryocytes, however, there was no increased granulopoiesis; in only 52 spleens was a high megakaryocyte count associated with marked granulocyte production. Combination with marked erythropoiesis was found significantly more often (in 71 of 224 spleens), nut erythropoietic activity was absent in 63 spleens with abundant megakaryocytes. Lymphoid hyperplasia was never observed in our material. No relationship between megakaryocytes and other blood cells in the spleen was therefore demonstrable.

Cavitary congestive dilatations

Furth and Boon (1945) made mention of cavitary dilatations in the liver in mice bearing a transplanted granulosa-cell tumour. These congestive sinusoidal dilatations associated with granulosa-cell tumours producing increased blood volume have since also been found in the spleen, adrenals and ovaries (Furth and Sobel, 1946; Cliffton and Wolstenholme, 1949; Bali and Furth, 1949; Furth and Moshman, 1951). The same phenomenon was encountered in mice bearing a transplanted interstitial testicular tumour (Wolstenholme and Gardner, 1950). A correlation with increased blood volume was found. The findings of the above-mentioned authors were confirmed in that cavitary dilatations in the organs were only found in animals with increased blood volume. A correlation was found between the degree of the anomaly and the degree of increased blood volume. Slight dilatations—periportal in the liver and as a rule in the periphery of the adrenal cortex—became visible once the plasma volume had increased to about 1·50 × its original value; marked spongy changes occurred at about 3·75 × the normal plasma volume (Fig. 10). In 17 per cent of the animals with a plasma volume increased to more than twice its original value no congestive changes were seen. However, the ovaries were not examined and the anomaly was sometimes confined to a single organ. No other correlation than that with the increased blood volume was found for the sinusoidal congestive dilatations.

EXPLANATION OF PLATES.

FIG. 4.—Granulocytes in lung. × 475. Mature nuclei hypersegmented and looped. Salivary gland tumour BDf (34th transplantation). Tumour weight: 7·8 g. Leucocyte count in peripheral blood computed on normal blood volume: 106,300/c.mm.

FIG. 5.—Immature granulocytes in liver. × 475. Testicular tumour DB (24th transplantation). Tumour weight: 9·3 g. Leucocyte count in peripheral blood computed on normal blood volume: 490,500/c.mm.

FIG. 6.—Granulocytopoiesis in spleen. × 475. Mature nuclei mostly closed-ring-shaped. Testicular tumour DB (28th transplantation). Tumour weight: 6·3 g. Leucocyte count in peripheral blood computed on normal blood volume: 53,600/c.mm.

FIG. 7.—Granulocytopoiesis within medullary cords of lymph node. × 475. Shapes of mature nuclei: closed-rings, open-serrated-rings and loops. Testicular tumour DB (24th transplantation). Tumour weight: 6·8 g. Leucocyte count in peripheral blood computed on normal blood volume: 55,400/c.mm.

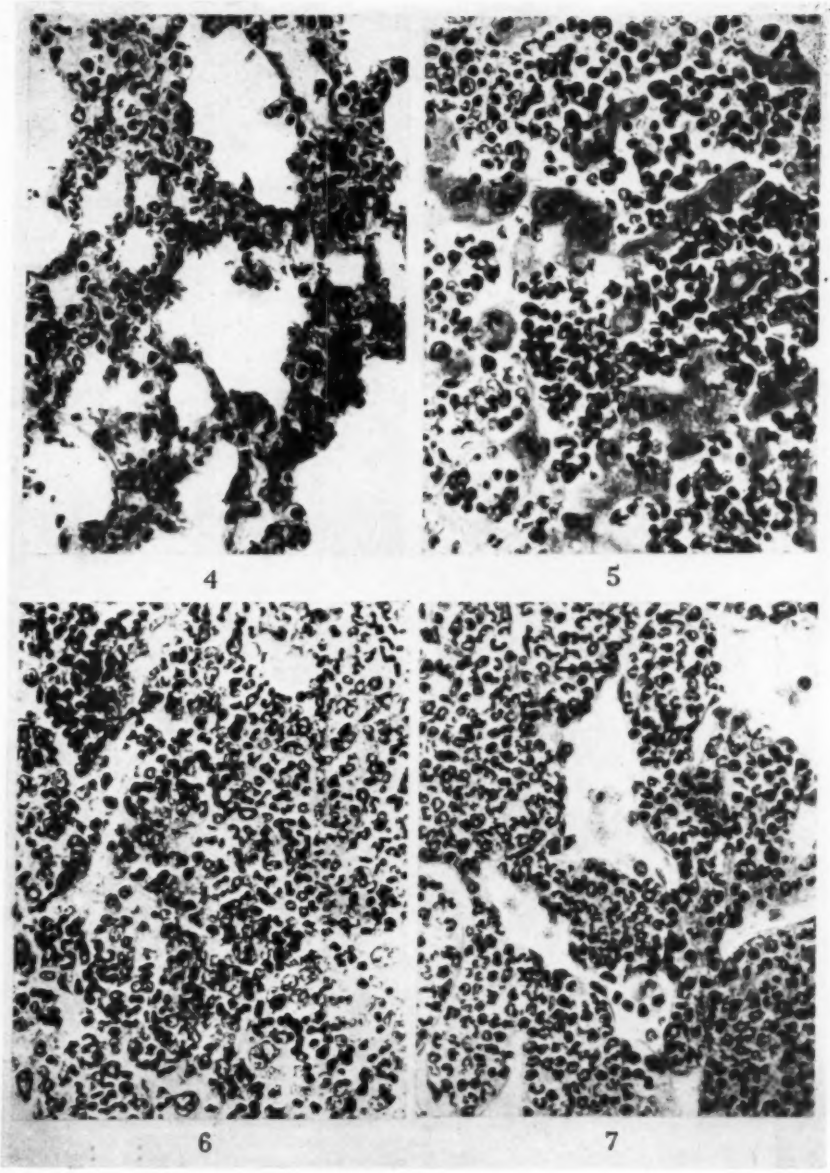
FIG. 8.—Erythropoiesis in liver. × 250. Nucleated red cells within the capillaries. Adrenal cortical carcinoma DO (14th transplantation). Tumour weight: 5·6 g.
Blood counts computed on normal blood volume

Haematocrit . .	0·68 × normal value.
R.B.C./c.mm. . .	0·35 × „ „
Cell volume μ^3 . .	1·93 × „ „
Haemoglobin . .	0·56 × „ „
Hb concentration .	0·79 × „ „

FIG. 9.—Erythropoiesis in kidney. × 250. Nucleated red cells within the glomerulus. Testicular tumour Af (13th transplantation). Tumour weight: 4·2 g.
Blood counts computed on normal volume:

Haematocrit . .	2·06 × normal value.
R.B.C./c.mm. . .	1·69 × „ „
Cell volume μ^3 . .	1·27 × „ „
Haemoglobin . .	1·69 × „ „
Hb concentration .	0·92 × „ „

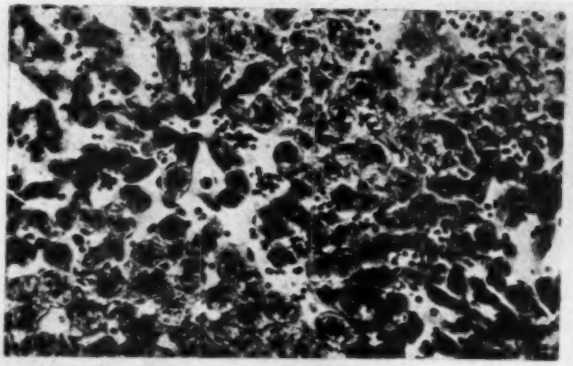
FIG. 10.—Sinusoidal dilatation in liver. × 250. Immature granulocytes and nucleated red cells within sinusoids. Testicular tumour DB (19th transplantation). Plasma volume: 4·74 × normal value. Tumour weight: 7·5 g.



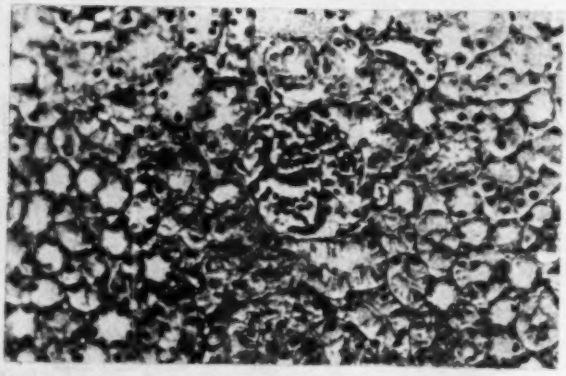
4 5

6 7

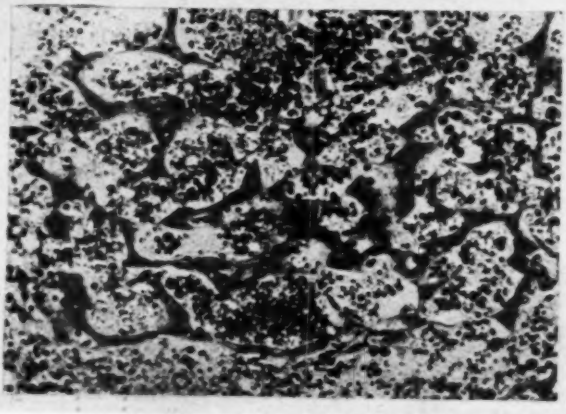
Tengbergen and Mühlbock.



8



9



10

Tengbergen and Mühlbock.

SUMMARY

An investigation was made into the effect of spontaneous and transplanted tumours on the blood in mice of various strains. The material investigated included 1264 tumour mice, of which 31 had spontaneous mammary tumours, while 1233 were bearing transplanted tumours of varying origin. These tumours, to a total of 76, included 44 mammary tumours, 2 interstitial testicular tumours, 5 adrenocortical tumours, 5 hepatomata, 2 squamous gastric tumours, 3 maxillary sarcomatoid carcinomata, 1 mixed tumour of the salivary glands, 3 pulmonary tumours, 4 uterine sarcomata, 3 fibro-sarcomata, 2 myxosarcomata and 2 haemangiomata.

The interpretation of haematological values in the tumour mice was based on determinations of the normal blood volume in 1840 untreated mice of the same strains as the tumour mice. The normal blood volume proved to be dependent on the strain, the body weight and sometimes on the sex of the animals.

Many tumour mice proved to have increased blood volumes, as a rule plasmatic but sometimes also polycythaemic or exclusively so. After correction of the cell and Hb values found (by conversion based on the normal blood volume), the tumour mice were found to have one or more of the following disorders: haemolytic anaemia, symptomatic polycythaemia and leukaemoid reaction.

Splenectomy had no inhibitory or stimulating effect on any of the haematological anomalies.

Causative factors were studied; necrosis of the tumour was found to be of little or no importance in this respect; nor was the size of the tumour significant. Sex hormones produced by the tumours probably constituted no causative, but may be a promoting, factor for increased blood volume and polycythaemia.

Morphological examination of the haemopoietic organs revealed granulopoiesis in accordance with the degree of leucocytosis of the peripheral blood. Erythropoiesis corresponded to the degree of anaemia but afforded no explanation of the polycythaemia.

An increase in the megakaryocyte count of the spleen in tumour mice was not associated with a parallel marked degree of granulopoiesis or erythropoiesis.

In the granulopoietic organs a predominance was found of a myeloid-cellular nuclear type typical of each organ.

The usually moderate splenomegaly in tumour mice was attributable chiefly to increased haemopoiesis.

Congestive sinusoidal dilatations in the liver and the adrenals were only found in animals showing increased blood volume.

REFERENCES

Bali, T. and Furth, J.—(1949) *Cancer Res.*, **9**, 449.
Barnes, W. A. and Sisman, J. E.—(1939) *Amer. J. Cancer*, **37**, 1.
Bateman, J. C.—(1951*a*) *J. nat. Cancer Inst.*, **11**, 671.—(1951*b*) *Blood*, **6**, 639.
Bruyn, W. M. de, Korteweg, R. and Waveren, E. Kits, van.—(1949) *Cancer Res.*, **9**, 282.
Burke, J. D.—(1954) *Phys. Zool.*, **27**, 1.
Clifton, E. E. and Wolstenholme, M. D.—(1949) *Cancer Res.*, **9**, 331.
Contopoulus, A. N., Ellis, S., Simpson, M. E., Lawrence, J. H. and Evans, H. M. —(1954) *Endocrinology*, **55**, 808.

COURTICE, F. C.—(1943) *J. Physiol.*, **102**, 290.
DUNN, TH. B.—(1954) *J. nat. Cancer Inst.*, **14**, 1281.
DYKE, D. C. VAN, CONTOPOULUS, A. N., WILLIAMS, B. S., SIMPSON, M. E. AND LAWRENCE, J. H.—(1954) *Acta haemat.*, **11**, 203.
FURTH, J.—(1946) *Proc. Soc. exp. Biol. N.Y.*, **61**, 212.
Idem, BEALE, E. J., WISH, L. AND KNOOHUIZEN, M. M.—(1951) *Cancer Res.*, **11**, 249.
Idem AND BOON, M. C.—(1945) *Proc. Soc. exp. Biol. N.Y.*, **58**, 112.
Idem AND MOSHMAN, J.—(1951) *Cancer Res.*, **11**, 543.
Idem AND SOBEL, H.—(1946) *J. nat. Cancer Inst.*, **7**, 103.
GOTTSCHALK, R. AND FURTH, J.—(1951) *Acta haemat.*, **5**, 100.
GUSSIO, S.—(1911–12) *Tumori*, **1**, 661.
HAM, A. W.—(1950) 'Histology'. Philadelphia (Lippincott).
HENSLER, L.—(1953) *Schweiz. med. Wschr.*, **83**, 1032.
KLEMPERER, P.—(1938) 'Handbook of Hematology'. New York (Hoeber), p. 1591.
MÜHLBOCK, O.—(1952) *Geburtsh. u. Frauenheilk.*, **12**, 289.
NIE, R. VAN.—(1957) Thesis, Utrecht.
PAPPENHEIM, A.—(1911) *Folia haemat.*, **10**, 393.
PETRI, S.—(1933) *Acta path. microbiol. scand.*, **10**, 159.
PONDER, E. AND NESMITH, J.—(1952) *Cancer Res.*, **12**, 104.
QUERIDO, A. AND OVERBEEK, A.—(1938) *Arch. int. Pharmacodyn.*, **59**, 370.
RUSSELL, E. S., NEUFELD, E. F. AND HIGGINS, C. T.—(1951) *Proc. Soc. exp. Biol. N.Y.*, **78**, 761.
SOBEL, H. AND FURTH, J.—(1948) *Endocrinology*, **42**, 436.
WEISBERGER, A. S., QUYTON, R. A., HEINLE, R. W. AND STORAASLI, J. P.—(1951) *Blood*, **6**, 916.
WISH, L., FURTH, J. AND STOREY, R. H.—(1950) *Proc. Soc. exp. Biol. N.Y.*, **74**, 644.
WOLSTENHOLME, J. T. AND GARDNER, W. U.—(1950) *Ibid.*, **74**, 659.

SERUM PROTEIN AND GLYCOPROTEIN CHANGES DURING GROWTH OF EXPERIMENTAL TUMOURS IN THE RAT

R. W. BALDWIN AND H. J. HARRIES

From the Cancer Research Laboratory, The University, Nottingham

Received for publication January 18, 1958

NUMEROUS studies have revealed that the concentrations of the carbohydrate-containing proteins in serum are increased above their normal levels in patients suffering from cancer and also in animals bearing experimental tumours (Winzler, 1953; Greenspan, 1954; Lockey, Anderson and Maclagan, 1956; Almquist and Lausing, 1957). Although the observed changes are not considered to be specific for cancer since similar variations have been detected in a variety of clinical and experimentally induced pathological conditions (Weimer and Moshin, 1952; Greenspan, 1954; Winzler, 1955; Stary, 1957) considerable interest still exists regarding the origin and function of serum glycoproteins and factors responsible for their alteration in disease.

Apparently incompatible hypotheses have been proposed to explain the increase of serum glycoprotein which occurs during tumour growth. From studies of the serum protein and glycoprotein changes occurring in chronic inflammatory and malignant disease in man (Seibert, Seibert, Atno and Campbell, 1947) and during growth of transplanted tumour in mice (Catchpole, 1950), it was concluded that increases of serum glycoproteins occurred as a result of tissue degeneration. In contrast, Shetlar, Erwin and Everett (1950) have proposed a completely opposite hypothesis, namely that serum glycoproteins are produced during processes involving tissue proliferation, in order to explain their findings on the serum glycoprotein changes occurring during early stages of growth of the Walker carcinoma in the rat. A considerable amount of evidence has been reported in support of both hypotheses whilst studies of the serum glycoprotein changes during experimentally induced lesions which result in tissue proliferation so far have failed to resolve the problem (Shetlar, Bryan, Foster, Shetlar and Everett, 1949; Heppleston and Keyser, 1957).

The present investigation was undertaken in order to determine the serum protein and glycoprotein changes occurring during growth of transplanted and carcinogen-induced tumours in the rat. Changes in total serum protein concentration and composition, total serum glycoprotein (protein-bound carbohydrate) and serum mucoprotein (protein and carbohydrate) have been determined at various stages of tumour growth. Thus it has been possible to relate serum glycoprotein increases to the growth activity of the tumour and also to correlate the changes occurring in the various carbohydrate-containing protein fractions. A preliminary report of these findings has already been published (British Empire Cancer Campaign, Annual Report, 1955).

MATERIALS AND METHODS

Transplanted tumour studies

The tumour used in these experiments was a transplanted sarcoma (S66) originally induced with methylcholanthrene in inbred Wistar rats (Baldwin, 1955). This tumour, which was used between the 19th and 36th generation of transfer, grew readily in all implanted rats and no regressions have ever been recorded.

Inbred Wistar rats (60 to 90 days old) of both sexes were implanted subcutaneously in the right dorsal region with standard amounts of tumour taken from a single donor. At intervals after tumour implantation, groups of 4 to 6 rats were sacrificed by exsanguination from the heart under ether anaesthesia. Total body weight was determined and then tumours were excised and weighed. From these data, tumour growth was assessed as tumour weight and as

$$\frac{\text{tumour weight}}{\text{residual body weight}}$$

(Total body weight — tumour weight).

Carcinogen-induced tumour studies

Inbred Wistar rats of both sexes (60 to 80 days old) were injected subcutaneously in the right axillary region with a single injection of methylcholanthrene (3 mg./0·5 ml.) in olive oil. Animals were examined twice weekly and tumour size was assessed as the product of the three dimensions of the tumour which were determined with the aid of callipers. Sera were examined from three groups of rats in which the induced tumours corresponded approximately in size with 7-, 14- and 21-day-old implants of the transplanted tumour.

Chemical determinations

All chemical analyses were performed in duplicate using a 1/20 dilution of serum in 0·15 m. NaCl solution.

Total serum protein was determined using the biuret method of Weichselbaum (1946).

Total serum glycoprotein (Serum protein-bound carbohydrate).—Serum protein was precipitated with ethanol as described by Weimer and Moshin (1952), and the carbohydrate content of the precipitated protein was determined by the tryptophane procedure (Shetlar, Foster and Everett, 1948) using a standard containing an equimolar mixture of galactose and mannose.

Serum mucoprotein was isolated using the procedure of Weimer and Moshin (1952) and analysed for carbohydrate and protein.

Carbohydrate was determined using the tryptophane procedure.

Protein was estimated using the Folin-Ciocalteau phenol reagent (Kabat and Mayer, 1948). Although the results are expressed in terms of tyrosine content, it should be stressed that the Folin reagent is not specific for tyrosine groups in protein.

Electrophoretic technique

Zone electrophoresis was carried out in an apparatus which was a modification of that described by Franglen, Martin and Treharne (1955); i.e. with a horizontal

freely suspended paper in a moist chamber. Electrophoretic separation of undiluted serum (40 μl.) on Whatman No. 3MM. paper was carried out for 24 hours under a potential gradient of 4·0 volts/cm. in barbitone buffer (pH 8·6 ; I, 0·1). Proteins were located on the paper by staining with bromphenol blue (Hardwicke, 1954) and protein distribution curves were obtained from analyses of eluted, protein-bound dye. Protein distribution curves were resolved into components by the method of Pedersen (1940) and the relative composition was determined from area measurements.

RESULTS

Transplanted tumour

These results were obtained from three separate experiments in which sera were analysed at intervals during growth of subcutaneous implants of sarcoma S66 at the 21st, 23rd and 36th generation of transfer. Although the data have been combined, it must be emphasised that the serum changes observed in each experiment were essentially the same. More important however was the fact that the relationship of serum glycoprotein changes to tumour growth was identical in each experiment. The growth curve of the tumour (Fig. 1) indicates three phases of growth. Immediately following implantation, there was a period of slow growth during which time tumour grafts were becoming established. This was followed by a period of rapid growth starting between the 10th and 14th day after implantation and then finally as tumours became large, there was a gradual decrease in the rate of growth.

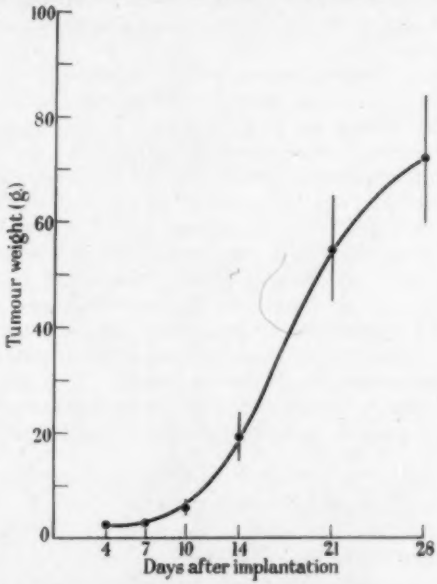


FIG 1.—Growth curve for tumour S66.

The serum protein and glycoprotein changes occurring during growth of sarcoma S66 are shown in Table I. Total serum protein (expressed as g./100 ml. serum) decreased steadily during tumour growth. Electrophoretic analyses of the sera (Table II) indicated that this loss of protein represented a loss of albumin together with β and γ globulins. The concentration of α_1 globulin was maintained at the normal level whilst the levels of α_2 globulins were slightly elevated immediately following tumour implantation and again at about the time when tumours began to grow rapidly. Because of the steady loss of total serum protein during tumour growth, the maintenance of the α globulins at or slightly above their normal levels results in an increase in the relative concentration of these proteins in serum.

Increases of serum mucoprotein, expressed either as protein (tyrosine) or carbohydrate, occurred immediately following tumour implantation and then after a period of little change, there was a further increase to a maximum. This second increase occurred whilst tumours were still comparatively small (3 to 10 per cent of total body weight) and could be correlated with the most active phase of tumour growth. The later stages of tumour development when tumours were becoming large (25–31 per cent of total body weight) were associated with decreases in the concentration of mucoprotein.

The composition of serum mucoprotein, expressed by its carbohydrate/protein (tyrosine) ratio, was not altered significantly by the large increases of mucoprotein occurring during the early stages of tumour growth. This suggests that the increases represent an abnormal production of the normal component. However, since the mucoprotein fraction isolated by precipitation with phosphotungstic acid from perchloric acid filtrates of serum contains a number of electrophoretically distinct proteins (Mehl, Humphrey and Winzler, 1949), the possibility remains that abnormal mucoproteins may be present in relatively minor amounts.

Total serum glycoprotein changes closely paralleled those of serum mucoprotein. However these changes mainly represent alterations in the concentration of the carbohydrate component of mucoprotein and the levels of serum glycoprotein other than mucoprotein (total serum glycoprotein–mucoprotein carbohydrate) steadily decreased during tumour growth. This was mainly due to the loss of serum protein and no significant changes were observed in the levels of the non-mucoprotein glycoprotein fraction when corrected for protein loss

$$\left(\frac{\text{Total serum glycoprotein} - \text{Mucoprotein carbohydrate}}{\text{Total serum protein}}\right).$$

Carcinogen-induced tumour

The results from these experiments are shown in Table III. Total serum protein concentration steadily decreased during tumour growth in a manner similar to that observed with transplanted tumour and the changes of non-mucoprotein glycoprotein could be accounted for by this loss of protein. In contrast, the concentration of serum mucoprotein was decreased during the early stages of tumour growth and only attained the normal level when the tumours had reached a relatively large size.

DISCUSSION

The electrophoretic studies (Table II) indicated that α_1 and α_2 globulins were maintained at or slightly above their normal levels during growth of trans-

TABLE I.—*Serum Protein and Glycoprotein Changes During Growth of Transplanted Tumour, S66, in the Rat*

		Tumour-bearing rats: Days after tumour implantation					
	Normal	4	7	10	14	21	28
Number of animals	12	6	15	5	15	14	9
Tumour weight (g.)	—	2·4±0·3	2·8±0·8	5·6±1·7	19·2±5·0	54·5±10·9	71·8±24·9
Tumour weight × 100 (%) / Residual body weight	—	1·4±0·1	1·5±0·6	2·6±0·2	9·8±3·3	25·2±5·9	31·1±9·4
Total serum protein (g./100 ml.)	6·29±0·10	5·66±0·18	5·84±0·27*	5·33±0·18	5·44±0·32*	5·19±0·22†	5·08±0·34
Total serum glycoprotein (mg./100 ml.)	195±4	218±8	205±10	206±17	255±24*	222±14†	207±21†
Serum mucoprotein—							
(*a*) Tyrosine (Tyr) (mg./100 ml.)	50±2	85±10	71±15*	83±7	105±21*	94±7	90±11
(*b*) Carbohydrate (CHO) (mg./100 ml.)	35±1	58±4	52±2*	60±19	82±16*	75±9	63±9†
Mucoprotein . $\frac{CHO}{Tyr}$	0·70±0·02	0·69±0·07	0·76±0·17	0·71±0·05	0·79±0·05	0·79±0·08	0·74±0·08
(Total serum glycoprotein-mucoprotein . CHO) mg./100 ml.	161±17	155±9	153±12	146±17	152±17	147±11	142±15
(Total serum glycoprotein-mucoprotein . CHO) ÷ Total serum protein × 100%	2·56±0·3	2·71±0·20	2·60±0·22	2·52±0·19	2·84±0·31	2·83±0·21	2·80±0·23

* P = < 0·01.
† *P* = 0·05–0·01.
(Results obtained from rats bearing 4- and 10-day-old tumours not included in statistical analysis.

TABLE II.—*Serum Protein Levels in Rats Bearing Transplanted Tumour, S66*

(Mean ± Standard Deviation)

Group	Number of animals	Albumin		Globulins							
				α_1		α_2		β		γ	
		(%)	(g. %)	(%)	(g. %)	(%)	(g. %)	(%)	(g. %)	(%)	(g. %)
Normal	12	42·8±0·6	2·69±0·07	19·6±0·6	1·23±0·04	11·3±0·3	0·71±0·03	16·9±0·03	1·06±0·02	9·2±0·6	0·58±0·05
Tumour-bearing rats. Days after implantation											
4	6	36·9±0·8	2·09±0·05	20·8±0·5	1·18±0·02	15·5±0·5	0·88±0·03	20·0±0·3	1·13±0·02	6·7±0·3	0·38±0·02
7	10	37·1*±3·4	2·12*±0·15	21·7±1·8	1·25±0·16	14·5*±1·9	0·83†±0·12	19·2*±0·9	1·11±0·08	7·6±1·4	0·45±0·09
10	5	35·6±0·9	1·90±0·07	22·1±0·9	1·18±0·04	14·5±1·0	0·77±0·06	19·9±0·3	1·06±0·02	7·9±0·6	0·42±0·04
14	10	32·7*±3·5	1·73†±0·19	23·6±2·1	1·24±0·11	17·0*±1·3	0·90±0·08	20·1±1·5	1·06±0·07	6·5±1·1	0·34±0·06
21	10	34·8±2·9	1·80±0·16	25·6*±1·6	1·32±0·08	14·4*±1·7	0·74*±0·09	19·4±1·4	1·00±0·10	5·9±0·6	0·31±0·04
28	5	36·7±1·0	1·84±0·05	25·1±0·7	1·27±0·05	14·2±0·6	0·73±0·04	17·5±0·2	0·88±0·02	6·6±0·4	0·34±0·02

* $P = < 0{\cdot}01$.

† $P = 0{\cdot}05$–$0{\cdot}1$.

(Results obtained from rats bearing 4, and 10-day-old tumours not included in statistical analysis.)

TABLE III.—*Serum Protein and Glycoprotein Changes During Growth of Methylcholanthrene-induced Tumours in the Rat*

(Mean ± Standard Deviation)

		Carcinogen-treated rats Days after injection		
	Normal	65–70	119–150	168–172
Number of animals	12	5	5	5
Tumour size (cu. cm.)	—	1·0–2·5	5·0–11·0	58–138
Total serum protein g./100 ml.	6·29±0·10	6·33±0·26	5·91±0·37	5·20±0·20
Total serum glycoprotein (T.CHO) mg./100 ml.	195±4	192±6	188±19	163±10
Serum mucoprotein—				
(*a*) Tyrosine (Tyr) mg./100 ml.	50±2	33±1	33±4	49±6
(*b*) Carbohydrate (CHO) mg./100 ml.	35±1	28±1	28±3	35±3
Mucoprotein . $\frac{\text{CHO}}{\text{Tyr}}$	0·70±0·02	0·83±0·03	0·88±0·05	0·72±0·02
(Total serum glycoprotein-mucoprotein CHO) mg./100 ml.	161±17	164±7	162±9	128±6
Total serum glycoprotein-mucoprotein . CHO ÷ Total serum protein × 100%	2·56±0·03	2·59±0·12	2·54±0·27	2·46±0·09

planted tumour (S66) in the rat despite a steady loss of all other serum proteins. In agreement with recent findings on α globulin changes during growth of transplanted tumour in mice (Bernfeld and Homburger, 1955), increases of α_2 globulins occurred during early stages of tumour development when growth was becoming most active.

Recently, it has been shown that between 80 and 90 per cent of the serum mucoprotein component of normal and malignant rat serum is distributed between the α_1 and α_2 globulins (Baldwin and Harries, unpublished observations). Thus at least part of the α globulin changes occurring during tumour growth can be related to increases of serum mucoprotein. In addition, it has been shown that total serum glycoprotein changes can be accounted for almost entirely by changes of serum mucoprotein-carbohydrate. These findings suggest that the major changes occurring in the serum glycoprotein fractions during tumour growth are due to alterations of serum mucoprotein concentration.

The early increase of serum mucoprotein occurring immediately following tumour implantation almost certainly represents the host's response to the implantation procedure and the tumour graft. The second increase to a maximum following a period of little change occurred whilst tumours were still comparatively small in size (3 to 10 per cent of total body weight) and could be correlated with the beginning of the most active phase of tumour growth. It is concluded from these findings that abnormal production of serum mucoprotein can be related to processes occurring during tissue proliferation rather than to the elimination of tissue glycoproteins following degenerative changes in tumour and surrounding tissue (Catchpole, 1950). This conclusion is further supported by the finding that mucoprotein levels decreased during the later stages of tumour growth when tumours were becoming large.

These findings fully confirm recent observations of Hokkanen, Pyörälä and Taipale (1956) on the mucoprotein changes associated with growth of the I.T.B.

ascites tumour in the rat and also to a limited extent those of Weimer, Quinn, Moshin and Nishihara (1957) on the serum glycoprotein changes occurring during growth of the Walker carcinoma. Comparison of the findings of the latter authors with those obtained in the present study reveals a number of marked differences. Thus although serum mucoprotein increases were observed with both tumours at about the same stage of development, further growth of the Walker carcinoma was associated with a continued production of mucoprotein whereas in the present study, decreases towards the normal level occurred during the later stages of tumour growth. In addition, growth of the Walker carcinoma resulted in a marked decrease in the concentration of serum glycoprotein other than mucoprotein, which could not be accounted for by loss of serum protein, whilst in the present study, normal or slightly elevated levels were detected. It is probable that these variations reflect differences in the growth requirements of the tumours since Weimer, Quinn, Moshin and Nishihara (1957) showed that even alteration of the site of implantation of the Walker carcinoma resulted in changes in the serum glycoprotein pattern.

Serum glycoprotein changes occurring during growth of methylcholanthrene-induced tumours differed from those observed with transplanted tumour in that there was a decrease rather than an increase of mucoprotein during the early stages of tumour growth. The activity of the induced tumours, expressed in terms of their growth rate, was very much less than that of the transplanted tumour S66 and so these results are interpreted as indicating that serum mucoproteins are only elevated during rapid tumour proliferation. The present findings therefore support the concept that serum mucoprotein increases during tumour growth occur as a result of proliferative processes and it is also suggested that these increases are related to the growth activity of the tumour.

SUMMARY

1. Total serum protein concentration steadily decreased during growth of transplanted tumour, S66 in the rat. This loss of protein represented a loss of all serum proteins except α_1 and α_2 globulins. Increases in α_2 globulin concentration occurred immediately after tumour implantation and again during the period of rapid tumour growth whilst α_1 globulins were maintained at their normal level.

2. Changes of serum mucoprotein concentration closely paralleled those observed for α_2 globulins: the major increase occurring during the most active period of tumour growth. Later stages of tumour development were associated with decreases in concentration of serum mucoprotein.

3. Total serum glycoprotein changes could be accounted for almost entirely by the alterations in serum mucoprotein-carbohydrate. The non-mucoprotein component corrected for loss of serum protein,

$$\left(\frac{\text{Total serum glycoprotein} - \text{Mucoprotein CHO}}{\text{Total serum protein}}\right)$$

showed no significant change during tumour growth.

4. Changes occurring during growth of methylcholanthrene-induced tumours were different from those observed with transplanted tumour: the major finding

being a decrease rather than an increase of serum mucoprotein. The implications of these findings are discussed.

This work was supported by the Nottinghamshire Council of the British Empire Cancer Campaign.

REFERENCES

ALMQUIST, P. O. AND LAUSING, E.—(1957) *Scand. J. clin. Lab. Invest.*, **9**, 179.
BALDWIN, R. W.—(1955) *Brit. J. Cancer*, **9**, 646.
BERNFELD, P. AND HOMBURGER, F. (1955) *Cancer Res.*, **15**, 359.
CATCHPOLE, H. R.—(1950) *Proc. Soc. exp. Biol. N.Y.*, **75**, 221.
FRANGLEN, G. T., MARTIN, N. H. AND TREHARNE, J. D.—(1955) *J. clin. Path.*, **8**, 144.
GREENSPAN, E. M.—(1954) *Arch. intern. Med.*, **93**, 863.
HARDWICKE, J.—(1954) *Biochem. J.*, **57**, 166.
HEPPLESTON, A. G. AND KEYSER, J. W.—(1957) *Brit. J. exp. Path.*, **38**, 111.
HOKKANEN, E., PYÖRÄLÄ, K. AND TAIPALE, E.—(1956) *Acta path. microbiol. scand.*, **39**, 15.
KABAT, E. A. AND MAYER, M. M.—(1948) 'Experimental Immunochemistry'. Springfield, Ill. (C. C. Thomas), p. 321.
LOCKEY, E., ANDERSON, A. J. AND MACLAGAN, N. F.—(1956) *Brit. J. Cancer*, **10**, 209.
MEHL, J. W., HUMPHREY, J. AND WINZLER, R. J.—(1949) *Proc. Soc. exp. Biol. N.Y.*, **72**, 106.
PEDERSEN, K. O.—(1940) 'The Ultracentrifuge'. London (Oxford University Press), p. 296.
SEIBERT, F. B., SEIBERT, M. V., ATNO, A. J. AND CAMPBELL, H. W.—(1947) *J. clin. Invest.*, **26**, 90.
SHETLAR, M. R., FOSTER, J. V. AND EVERETT, M. R.—(1948) *Proc. Soc. exp. Biol. N.Y.*, **67**, 125.
Idem, BRYAN, R. S., FOSTER, J. V., SHETLAR, C. L. AND EVERETT, M. R.—(1949) *Ibid.*, **72**, 294.
Idem, ERWIN, C. P. AND EVERETT, M. R.—(1950) *Cancer Res.*, **10**, 445.
STARY, Z.—(1957) *Clin. Chem.*, **3**, 557.
WEICHSELBAUM, T. E.—(1946) *Amer. J. clin. Path.*, **16** (Tech. Suppl. **10**, 40).
WEIMER, H. E. AND MOSHIN, J. R.—(1952) *Amer. Rev. Tuberc.*, **68**, 594.
Idem, QUINN, F. A., MOSHIN, J. R. AND NISHIHARA, H.—(1957) *J. nat. Cancer Inst.*, **19**, 409.
WINZLER, R. J.—(1953) *Advanc. Cancer Res.*, **1**, 503.
NEW YORK (ACADEMIC PRESS).—(1955) 'Methods of Biochemical Analysis'. New York (Interscience), **2**, 279.

THE METABOLISM OF AROMATIC AMINES IN RELATION TO CARCINOGENESIS

L. A. ELSON, F. GOULDEN* AND F. L. WARREN†

From the Chester Beatty Research Institute, The Royal Cancer Hospital, London, S.W.3

Received for publication December 12, 1957.

SOME years ago, in connection with work on the metabolism of carcinogenic azo compounds, the method of excretion by the rat of a number of aromatic amines was studied. A preliminary account of some of this work was given at a meeting of the Biochemical Society (Elson, Goulden and Warren, 1946).

Many aromatic amines are excreted as ethereal sulphates of amino phenols, and one of the aims of this work was to investigate the effects of the amines on sulphur metabolism as had been done previously for a series of aromatic hydrocarbons (Elson, Goulden and Warren, 1945). The investigation was concerned therefore mainly with determining changes in the daily excretion of ethereal sulphate, inorganic sulphate, neutral sulphur and of glucuronic acid in the urine following administration of the aromatic amines.

It was hoped eventually to investigate more completely the metabolism of individual amines and to characterise definitely their metabolites, but this extended investigation has not been possible in most cases. The subsequent discovery of of the carcinogenic action in rats of a number of the amines, e.g. aminostilbenes (Haddow, Harris, Kon and Roe, 1948 ; Elson, 1952*a*), 4-dimethylamino-diphenyl (Miller, Miller, Sandin and Brown, 1949), benzidine (Spitz, Maguigan and Dobriner, 1950), and, in particular, 4-amino-diphenyl and 3 : 2′-dimethyl-4-aminodiphenyl by Walpole, Williams and Roberts (1952) and their suggestion that *ortho* hydroxyamines may be direct-acting carcinogens, has suggested the possibility of an interesting relationship between our findings for the metabolism of these aromatic amines and their carcinogenic action.

The purpose of the present paper therefore is to describe in more detail the results of our metabolism studies and to discuss their possible significance in relation to carcinogenesis.

EXPERIMENTAL

Animals

The experiments were carried out on male rats maintained on a high protein diet (diet A, Elson 1952*a*). Groups of six rats were kept in one metabolism cage and supplied with diet and water *ad libitum*. The daily excretion of glucuronic acid, inorganic sulphate, ethereal sulphate, and in most cases neutral sulphur was determined as described by Elson, Goulden and Warren (1945). This was done for several days before administration of the amines dissolved in arachis

* Present address : Ashford Hospital, Middlesex.
† Present address : London Hospital Medical College, E.1.

oil, by intraperitoneal injection and the estimations were continued for several days subsequently. In some cases, 1 ml. arachis oil was injected prior to the control period of estimations preceding administration of the amine, but as this was found to give no appreciable changes in amounts of the excretion components estimated it was discontinued.

Detection of free and conjugated amines

In many cases the excretion of the amine was followed by a colorimetric method for detection of free aromatic amino groups by diazotisation and coupling with N-sulphato-ethyl-m-toluidine, as described by Elson and Warren (1944). By the use of the " Spekker " photoelectric absorptiometer for measuring the colour obtained with the untreated urine and the colour obtained after acid hydrolysis of the urine, it was often possible to obtain an idea of the amount of free and conjugated amine present. (An increase in the colour test after hydrolysis probably indicates that some of the original amine has been acetylated and excreted as the acetyl amino derivative, as is known to occur with drugs such as sulphonamides, etc.)

RESULTS

The output of ethereal sulphates and glucuronides in the urine following injection of aromatic amines is shown in Fig. 1. With several of the amines the effect of more than one dose was investigated, and with some of them a large dose resulted in excretion of both ethereal sulphates and glucuronides. When the dose was reduced however, it was generally found that an increase of ethereal sulphate alone, or of glucuronide alone occurred, so that with moderate doses the amine showed a preferential excretion either as ethereal sulphate or as glucuronide. Fig. 1 shows the results obtained with these " preferential " doses.

A typical case where increased dosage results in excretion of glucuronide in addition to the " preferential " ethereal sulphate is shown in Fig. 2 which gives the results of acetanilide at doses of 20mg./100g. rat. and 9mg./100g. rat. The inorganic sulphate excretion is also shown and clearly demonstrates the fall in inorganic sulphate corresponding to the rise of ethereal sulphate.

Table I shows the mode of excretion of the amines in relation to their carcinogenic properties. The probable nature of the principal metabolic products is also suggested. In a number of cases the unconjugated metabolic products have been isolated and characterised by various workers. The other suggestions are based on general inferences considered in conjunction with the present results. The percentage figures under some of the formulae refer to the amine estimated colorimetrically by diazotisation and coupling with N-sulphato-ethyl-m-toluidine. Increase in the intensity of this colour test after hydrolysis probably indicates acetylation, and from the values before and after hydrolysis an estimate of the relative percentage of free and acetylated amine present in the original urine has been obtained.

From Fig. 1 it is seen that the amines fall clearly into two groups. The first group consists of compounds containing a single benzene ring, viz. aniline, 4-chloroaniline, phenetidine, dimethylaniline, and acetanilide, which are excreted preferentially as ethereal sulphates. The second group consists of amines excreted preferentially as glucuronides, and these are the ones containing two

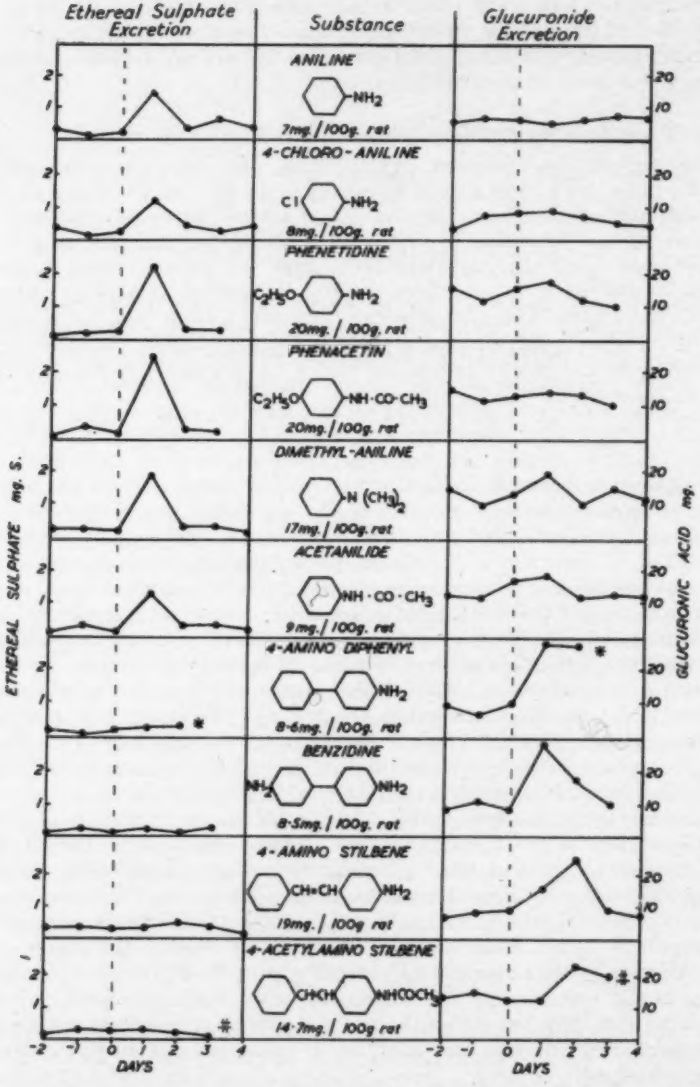


FIG. 1.—Ethereal sulphate and glucuronide excretion in rats following administration of aromatic amines.

*Toxic—1 rat died 3rd day after injection.
#Toxic—2 rats died 4th day after injection.

benzene rings, viz. 4-amino-diphenyl, benzidine, 4-amino-stilbene, 4-acetylamino-stilbene.

When the investigation was first started only 4-amino-stilbene had been shown to produce tumours in the rat. Now however, benzidine has been shown to be carcinogenic in this species (Spitz *et al.* 1950), dimethylamino-diphenyl was found to be carcinogenic by Miller *et al.*, (1949), and finally rat tumours have been induced with 4-amino-diphenyl by Walpole *et al.* (1952). This compound has also been shown to produce bladder tumours in dogs (Walpole, Williams and Roberts, 1954). It " appears to be more effective as a bladder carcinogen in the

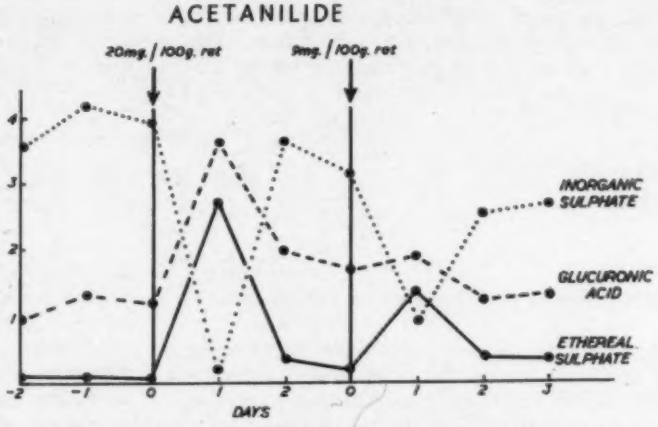


FIG. 2.—Excretion of inorganic sulphate, glucuronic acid and ethereal sulphate in rats treated with acetanilide.

Ordinate numbers represent mg.S for inorganic sulphate and ethereal sulphate; each figure multiplied by 10 gives the amount of glucuronic acid.

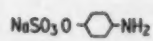
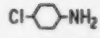
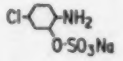
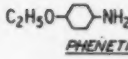
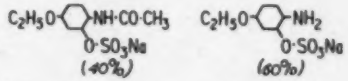
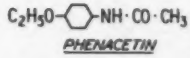
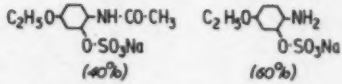
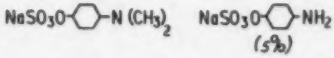
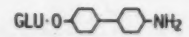
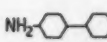
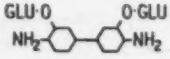
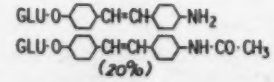
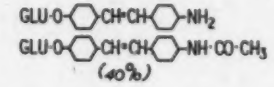
dog than either benzidine or 2-acetylamino fluorene and at least as potent as β-naphthylamine ". In contrast, none of the aniline derivatives comprising the first group (Fig. 1) (Table I) has been found to be carcinogenic.

DISCUSSION

Considering the mode of action of the carcinogenic aromatic amines it seems most probable that it is their metabolites, produced during the course of the so-called " detoxication " mechanism in the body, which constitute the real carcinogenic agents. Perhaps some of the most convincing evidence for this view is the failure of 2-naphthylamine, known to be responsible for production of bladder tumours in man, to produce tumours when implanted into the bladders of mice whereas its metabolite 2-amino-1-naphthol readily induces such tumours (Bonser, Clayson, Jull and Pyrah, 1952).

In their work on the carcinogenic action of 4-amino-diphenyl and, more particularly of 3 : 2′-dimethyl-4-amino-diphenyl, Walpole *et al.* (1952) suggested

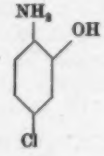
TABLE I.—*Mode of Excretion of Aromatic Amines in Relation to Carcinogenesis.*

AMINE	DOSE mg/100g rat.	EXCRETION AS Ethereal sulphate.	Glucuronic acid.	PROBABLE NATURE OF PRINCIPAL METABOLIC PRODUCTS.
ANILINE	7·25 14·0	+ ++	– slight	
 4-CHLOROANILINE	7·8	+	–	
 PHENETIDINE	20	++	–	 (40%) (60%)
 PHENACETIN	20	++	–	 (40%) (60%)
DIMETHYL ANILINE	17	++	slight	 (5%)
4-AMINO DIPHENYL	8·6 16	– +	++ ++	
 BENZIDINE	8·5	–	++	
4-AMINO STILBENE	19	–	++	 (20%)
4-ACETYLAMINO STILBENE	14·7	–	++	 (40%)

* CARCINOGENIC GLU = Glucuronic acid

that *ortho* hydroxy amines may be direct-acting carcinogens, and Clayson (1953) has put forward a working hypothesis for the mode of carcinogenesis of aromatic amines which suggests that "(1) compounds which contain a hydroxyl and an amino group *ortho* to each other in an aromatic system of two or more rings may be carcinogenic either in their own right or as a result of their further reaction; (2) the reason why some aromatic amines induce tumours whereas others do not is that the former are more readily converted in the body to *ortho*-hydroxy amines than the latter".

It must be emphasised that this remains merely an hypothesis and indeed Clayson himself points out the need for further investigation and experimental work both biological and biochemical. There is in fact, no evidence to suggest that *ortho* hydroxylation is the only essential for carcinogenicity in an aromatic amine, or that *para* hydroxy aromatic amines cannot be carcinogenic. It would for instance be extremely dangerous to assume that a blocked *para* position such as occurs in phenacetin and most probably leads to metabolic hydroxylation in the *ortho* position to the amino group could incur a suggestion of carcinogenicity in this drug. In fact Schmähl and Reiter (1954) in very prolonged feeding tests with rats during which a total dose of 22 g. of phenacetin was taken by each rat found no evidence of any carcinogenic effect of the drug. A similar case is represented by 4-chloro-aniline which has been shown by Elson and Spinks (unpublished) to be excreted in the rat and the rabbit as the *ortho* hydroxy derivative



and prolonged feeding of 4-chloro-aniline to rats failed to produce any evidence of its carcinogenicity.

Clearly other factors must also be involved in the carcinogenic potentialities of aromatic amines, and the results described in this paper, although they must be regarded rather in the nature of a preliminary approach to the problem, do at least suggest one other aspect, namely that not only the nature of the hydroxylated metabolite but also the method of conjugation may be of primary importance. In the limited series of aromatic amines examined a sharp change in the " preferential " conjugation of the amine from the ethereal sulphate to glucuronide occurs on passing from the simpler " one benzene ring " amines to the more complex " two benzene ring " amines. For the rat this change is accompanied by increased general toxicity and with the development of carcinogenic properties in the amines.

In considering the possible significance of preferential excretion as glucuronides, in this connection it was noted that 4-amino-phenol is excreted preferentially as glucuronide although aniline is excreted preferentially as the ethereal sulphate of 4-amino-phenol. It is not easy to see why, if the aniline is metabolised in the body to 4-amino-phenol it too should not be preferentially excreted as glucuronide, and one might be tempted to speculate that ethereal sulphate conjugation and oxidation could take place almost simultaneously without liberation of appreciable quantities of the toxic amino-phenol itself.

As for the significance of the glucuronide excretion, it has been suggested by one of us (Elson, 1952*b*) in connnection with the action of the enzyme glucuronidase that " glucuronic acid formation and the rôle of glucuronidase in animal metabolism may not be exclusively concerned with the so-called detoxication mechanisms, but may also be that of providing a transfer mechanism for conveyance of a fat-soluble but water insoluble substance such as a steroid hormone from one organ in the body to others on which it is required to act. The substance is transferred by being converted into a water soluble glucuronide, which may no

longer show hormone activity, and this is carried in the blood stream, and, in this water soluble form, is able to enter the cells of various organs. The active hormone is then liberated *in situ* in that organ by means of the glucuronidase present in the cell". The carcinogenic aromatic amines could thus become involved in this process in respect of their tendency to glucuronide formation, being transferred as their water soluble glucuronides into the cells of the various organs and the active carcinogen liberated by the glucuronidase present in the cells.

An indication of the possible significance of glucuronides in carcinogenesis is given by Boyland, Wallace and Williams (1955) who found that the ethereal sulphate of 2-amino-1-naphthol is not hydrolysed by the sulphatases of rat kidney, takadiastase, or human urine, whereas 2-amino-1-naphthyl glucosiduronic acid is hydrolysed by β-glucuronidase. The actual carcinogenic agent concerned in induction of cancer of the bladder in man by 2-naphthylamine is believed to be 2-amino-1-naphthol released from conjugates in the urine. The carcinogenic amino naphthol would not, according to their findings, be liberated from 2-amino-1-naphthol sulphuric ester but would be produced from 2-amino-1-naphthyl glucosiduronic acid. The glucuronide is therefore probably an essential intermediate metabolite in induction of bladder cancer by 2-naphthylamine, and this idea receives strong support from the findings of Allen, Boyland, Dukes, Horning, and Watson (1957) that of five metabolites of 2-naphthylamine tested by direct implantation in the bladders of mice, the only one to induce tumours was 2-amino-1-naphthylglucosiduronic acid.

SUMMARY

Aromatic amines administered to rats are excreted as ethereal sulphates or as glucuronides of amino phenols. Although with large doses increase in the urinary excretion both of ethereal sulphate and of glucuronic acid can occur, with lower doses a preferential excretion either as ethereal sulphate or as glucuronide is obtained.

The simpler "one benzene ring" aromatic amines, aniline, 4-chloroaniline, phenetidine, phenacetin, dimethylaniline, and acetanilide are excreted by the rat preferentially as ethereal sulphates whereas the "two benzene ring" amines, 4-aminodiphenyl, benzidine, 4-aminostilbene and 4-acetylaminostilbene are excreted preferentially as glucuronides.

All the amines of this latter group excreted as glucuronides have now been shown to be carcinogenic. Those of the former group, excreted as ethereal sulphates do not appear to be carcinogenic.

The possible significance of excretion as glucuronide in relation to carcinogenesis is discussed.

This work has been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund, and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

REFERENCES

ALLEN, M. J., BOYLAND, E., DUKES, C. E., HORNING, E. S. AND WATSON, J. G.—(1957) *Brit. J. Cancer*, **11**, 212.
BONSER, G. M., CLAYSON, D. B., JULL, J. W. AND PYRAH, L. N.—(1952) *Ibid.*, **6**, 412.
BOYLAND, E., WALLACE, D. M. AND WILLIAMS, D. C.—(1955) *Ibid.*, **9**, 62.
CLAYSON, D. B.—(1953) *Ibid.*, **7**, 460.
ELSON, L. A.—(1952*a*) *Ibid.*, **6**, 392.—(1952b) 'Ciba Foundation Colloquia on Endocrinology'. London (J. & A. Churchill), **1**, 284.
Idem, GOULDEN, F. AND WARREN, F. L.—(1945) *Biochem. J.*, **39**, 301.—(1946) *Ibid.*, **40**, XXIX.
Idem., AND WARREN, F. L.—(1944) *Ibid.*, **38**, 217.
HADDOW, A., HARRIS, R. J. C., KON, G. A. R. AND ROE, E. M. F.—(1948) *Phil. Trans.* A, **241**, 147.
MILLER, E. C., MILLER, J. A., SANDIN, R. B. AND BROWN, R. K.—(1949) *Cancer Res.*, **9**, 504.
SCHMÄHL, D. AND REITER, A.—(1954) *Arzneimittel Forsch.*, **4**, 404.
SPITZ, S., MAGUIGAN, W. H. AND DOBRINER, K.—(1950) *Cancer*, **3**, 789.
WALPOLE, A. L., WILLIAMS, M. H. C. AND ROBERTS, D. C.—(1952) *Brit. J. indust. Med.*, **9**, 255.—(1954) *Ibid.*, **11**, 105.

THE INTERMEDIARY METABOLISM OF PYRENE

K. H. HARPER

From the Department of Cancer Research, Mount Vernon Hospital and The Radium Institute, Northwood, Middlesex

Received for publication December 12, 1957.

In a previous communication to this journal (Harper, 1957) an investigation of the metabolic end products of pyrene following intravenous and intraperitoneal injections in the mouse and the rat was reported. These end products appeared in the urine and/or the faeces and were identified as : 3-hydroxypyrene, mainly free but possibly conjugated to a small extent, pyrene-3 : 8- and 3 : 10-quinones, and 3 : 8- and 3 : 10-dihydroxypyrenes mainly conjugated. In addition evidence was obtained of the presence of an acid-decomposable pyrene precursor and an unidentified " quinonoidal complex " which liberated free pyrene on heating with hydrochloric acid.

This work has now been extended to cover metabolism within the tissues and a report of the results and conclusions is given here.

MATERIALS AND METHODS

Throughout these experiments mice of RIII, Strong A, C.B.A. and Swiss strains were used and it is perhaps worthy of note that the same metabolic behaviour has been observed in all four strains.

The pyrene was prepared for injection as a fine colloidal dispersion containing 1 mg. per c.c. by dissolving 100 mg. in the smallest possible volume of acetone and injecting the acetone solution beneath the surface of 100 c.c. distilled water. The acetone was then removed from the resulting colloid under reduced pressure.

Each mouse received an intravenous injection of 0·5 c.c of the colloid. The animals in batches of 10 to 20 were then killed at intervals of $1\frac{1}{2}$ to $2\frac{1}{2}$ hours following the injection and the particular organ under consideration quickly dissected out and extracted immediately.

Based upon the isolation procedure devised for the intermediary metabolites of 3 : 4-benzpyrene by Weigert and Mottram (1946) and upon the properties of the pyrene derivative first isolated from the bile, the following general extraction procedure was adopted.

The organs were homogenised in a Waring blender, extracted with 70 per cent acetone and filtered. The acetone was then pumped off under reduced pressure and the residual aqueous phase saturated with ammonium sulphate and acidified with hydrochloric acid. Extraction with ether was then carried out until no appreciable fluorescent material was removed. The ether extract was dried with sodium sulphate, mixed with an equal volume of benzene and the ether removed from the mixture under reduced pressure. The benzene, approximately 200 c.c. in volume, was then chromatographed on columns of silica gel (100/200 mesh)

over alumina. These were best prepared by the slurry technique and separation of the two adsorbents was effected by the interposition of discs of filter paper. Small chromatography tubes were generally used, of internal diameter 14 mm. and length 16·5 cm. and contained approximately equal lengths of the two adsorbents.

RESULTS

(a) *Gall bladder*.—The bile possessed a strong blue fluorescence in ultraviolet light and for extraction purposes it was diluted with several volumes of distilled water.

An ether extract of the diluted bile showed a weak blue fluorescence and contained only a trace amount of free pyrene. After acidification with hydrochloric acid however, a further ether extract was yellow in colour and possessed a strong blue/violet fluorescence. Spectroscopic examination suggested that this was due to the presence of a pyrene derivative with its adsorption maxima displaced to

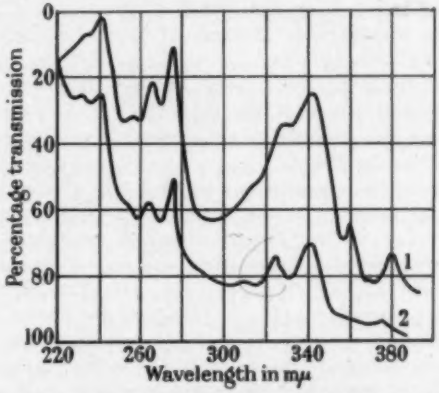


FIG. 1.—Absorption spectra in ethyl alcohol. 1. 3-Hydroxypyrene glucuronide conjugate. 2. PX.

shorter wavelengths relative to those of 3-hydroxypyrene. The ether extract was transferred to benzene which was then chromatographed on silica gel over alumina as described above. Examination of the chromatogram under ultraviolet light revealed the presence of a diffuse dull blue/violet zone extending downwards from the surface of the silica gel. This, when eluted with ethyl alcohol, gave a blue/violet fluorescent solution with the pyrene type spectrum referred to above. As eluted from the silica, however, it appeared to be associated with a certain amount of yellow colouring matter. It was finally obtained free from this by evaporating a solution in slightly moist ether to dryness and washing the residue with several small volumes of benzene. The residue, dissolved in ethyl alcohol, was colourless and possessed the absorption spectrum shown in Fig. 1 with maxima at 234, 242, 255, 265, 276, 328, 342, 359–60, 371 and 380 mμ.

The new derivative was readily soluble in water and on warming with dilute hydrochloric acid was rapidly converted into 3-hydroxypyrene.

With naphthoresorcinol reagent it gave a weak positive test for glucuronic acid.

In view of these properties allied with the close spectral similarity to 3-hydroxypyrene it appeared likely that the derivative was a glucuronide conjugate of 3-hydroxypyrene. This was verified by incubation at 37° C. with β-glucuronidase (bacterial) at pH 7 when cleavage of the glucosiduronic acid rapidly took place resulting in the formation of pure 3-hydroxypyrene.

(b) *Duodenum and small intestine.*—Spectroscopic examination of the ether phase during the extraction procedure suggested the presence of a considerable amount of the 3-hydroxypyrene glucuronide conjugate and this was confirmed on analysis of the silica gel chromatogram. In addition a small amount of free 3-hydroxypyrene was identified on the alumina.

(c) *Caecum and large intestine.*—Analysis of the chromatogram from these tissues revealed that the major metabolite was now 3-hydroxypyrene and that only a small amount of the glucosiduronic acid was present.

(d) *Bladder.*—Extraction of the urine with ether at pH 7 resulted in the removal of a small amount of free 3-hydroxypyrene. On adjusting the pH of the urine to pH 2, however, a considerable amount of the 3-hydroxypyrene glucuronide conjugate was extractable with ether.

(e) *Kidney.*—The fluorescence of the extract from this tissue was much reduced in intensity compared with that from the gut. Analysis of the chromatogram revealed that a small amount of the glucosiduronic acid and a trace amount of 3-hydroxypyrene were present.

(f) *Liver.*—Contrary to expectations neither the glucosiduronic acid nor the free phenol were extractable from this tissue. Instead however, on elution with ethyl alcohol, the silica gel yielded a straw coloured, weak blue fluorescent solution. This eluate, on spectroscopic examination, was found to possess a pyrene type spectrum with maxima at 232, 242, 264, 275, 310, 324-25, 340–41 and 376 mμ. A purer sample (spectrum Fig. 1) was obtained by direct chromatography on alimina when the derivative, symbolised as PX, was held as a narrow zone at the surface. The appearance of the spectrum in the lower wavelengths suggested the presence of strongly absorbing background material but attempts to remove such material were unsuccessful.

When a solution of the PX in ethyl alcohol was acidified with hydrochloric acid the fluorescence rapidly changed to blue/violet and on heating the mixture the PX was converted into 3-hydroxypyrene. The same transformation was effected by incubation with β-glucuronidase so it is possible that a β-glucosiduronic linkage is present in the molecule. The general behaviour of this compound was therefore in keeping with it being a glucuronide conjugate of an unstable pyrene diol, but the spectroscopic evidence did not support this view.

The site of metabolism

In an attempt to determine the site of metabolism within the animal incubation experiments were carried out with liver and kidney slices. The slices were incubated at 37° C. for 3 hours in Tyrode solution containing 0·1 mg. colloidal pyrene per c.c., oxygen being bubbled through the solution to ensure adequate aerobic conditions. The slices were then subjected to the extraction procedure used for the *in vivo* experiments.

Liver slices.—The slices assumed a distinct blue/violet fluorescence and on

extraction the unidentified PX, 3-hydroxypyrene and the 3-hydroxypyrene glucuronide conjugate were identified.

Kidney slices.—The fluorescence of the slices remained unaltered and on extraction no evidence of metabolism was obtained.

DISCUSSION

In view of the fact that PX is the only metabolite extractable from the liver of the intact animal whereas the free phenol and the phenol glucuronide conjugate are obtained in addition to PX from liver slice incubation experiments, it appears likely that PX is the primary metabolite formed during the metabolic oxidation of pyrene to 3-hydroxypyrene. These experiments suggest that the PX is converted into the 3-hydroxypyrene glucuronide conjugate in the liver and that the latter is then immediately removed in the bile or transported to the kidney and eliminated in the urine. The hydrolytic cleavage of the glucuronide conjugate to the free phenol which then takes place, either during passage through the caecum and large intestine or during storage in the bladder, must be attributed to enzymatic activity as the glucuronide is only slowly decomposed by acid at 37° C.

Support for these conclusions is provided by the established presence of β-glucuronidase in the urine and by the findings of Marsh, Alexander and Levvy (1952) who determined the sites of glucuronide decomposition down the digestive tract of various animal species. Unfortunately the mouse was not included but in the rat, which yields the same metabolites of pyrene as the mouse (Harper, 1957) the concentration of glucuronide decomposing enzymes in the caecum and colon relative to that in the duodenum and ileum was approximately 30 : 1. In the rabbit the ratio was even higher, being 180 : 1.

The absence of the quinones in the large intestine suggests that these are formed as oxidation products of the 3-hydroxypyrene after excretion in the faeces.

In view of the finding that the urine obtained from the dissected bladder contained principally the glucuronide conjugate the apparent anomalous behaviour previously observed with 24 hour urine specimens can now be explained. It was found that after continuous Soxhlet extraction with ether at pH 6 to remove free phenol, the 24 hour urine sampler yielded a further amount of 3-hydroxypyrene during extraction with ether at pH 2, this being most marked in the case of the mouse (Harper, 1957). This can now be attributed to the hydrolysis of the glucuronide conjugate during the warm acidic conditions prevailing in the Soxhlet apparatus. Failure to obtain glucuronide conjugate as such from the urine has now been shown to be due to the isolation procedure adopted as the glucuronide is strongly adsorbed on alumina from benzene and cannot be eluted with ethyl alcohol. It can be eluted, however, if a small amount of hydrochloric acid is added to the alcohol.

Although providing a satisfactory explanation for the final elimination of free 3-hydroxypyrene in both urine and faeces these experiments have yielded no evidence concerning the presence of dihydroxy compounds in the urine. No trace of these compounds or possible metabolic precursors has been obtained from the tissues examined so it is possible that they, like the quinones, are formed as secondary oxidation products from 3-hydroxypyrene. This problem is to receive further investigation.

SUMMARY

(1) Evidence has been obtained that the metabolic oxidation of pyrene to 3-hydroxypyrene proceeds via two intermediate stages.

(2) The experiments suggest that a primary metabolite, symbolised as PX, is formed in the liver and that this is converted into the glucuronide conjugate of 3-hydroxypyrene which is excreted in the bile and urine.

(3) During storage in the bladder or passage through the caecum and large intestine the glucuronide conjugate is then enzymatically hydrolysed to free phenol.

(4) The nature of the metabolite PX has not yet been determined.

REFERENCES

HARPER, K. H.—(1957) *Brit. J. Cancer*, **11**, 499.
MARSH, C. A., ALEXANDER, F. AND LEVVY, G. A.—(1952) *Nature (Lond.)*, **170**, 163.
WEIGERT, F. AND MOTTRAM, J. C.—(1946) *Cancer Res.*, **6**, 97.

THE INTERMEDIARY METABOLISM OF 3 : 4-BENZPYRENE

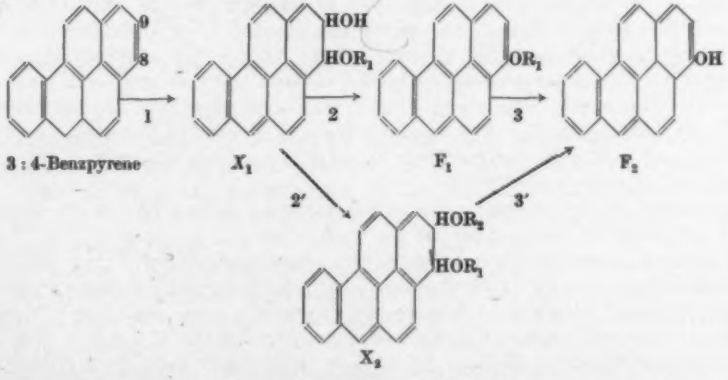
K. H. HARPER

From the Department of Cancer Research, Mount Vernon Hospital and the Radium Institute, Northwood, Middlesex

Received for publication December 12, 1957

FOLLOWING on from previous findings that 3 : 4-benzpyrene is metabolised to two distinct derivatives, BPX and BPF, which differ in chemical properties and fluorescence spectra (Chalmers and Peacock, 1936 ; Chalmers, 1938 ; Chalmers and Crowfoot, 1941), Weigert and Mottram (1946) succeeded in isolating four different metabolites symbolised as X_1, X_2, F_1 and F_2. The X derivatives only were extractable from the liver, bile and small intestine whereas the F derivatives were present as major metabolites in the large intestine and faeces. The F derivatives were also found when tissues containing the X metabolites were stored for any length of time. In view of the apparent metabolic transformation and chemical properties of the derivatives and by analogy with the known metabolic fate of the non-carcinogenic hydrocarbon, anthracene, they postulated the following oxidative mechanism.

The groups R_1 and R_2 were not identified but an acidic nature was postulated for them.



The metabolite F_2 proved to be identical with 8-hydroxy-3 : 4-benzpyrene previously identified as an end product in the faeces (Berenblum, Crowfoot, Holiday and Schoental, 1943).

This reasoning, however, has been criticised by Berenblum and Schoental (1955) who argue that the long wave systems of the absorption spectra of X_1 and X_2 are very similar to those of fully aromatic benzpyrene derivatives and that F_1 is indistinguishable from unconjugated 10-hydroxy-3 : 4-benzpyrene previously identified in faeces (Berenblum and Schoental, 1946).

In view of the finding that the metabolism of the non-carcinogenic hydrocarbon pyrene proceeds via tissue intermediates possessing the fully aromatic pyrenoid configuration (Harper, 1958) it seemed desirable therefore to re-examine the metabolism of 3 : 4-benzpyrene in the hope of resolving this controversy.

MATERIALS AND METHODS

Mice of RIII, Strong A, C.B.A. and Swiss strains were used for these experiments. Each mouse received an intravenous injection of 0·5 c.c. of a colloidal dispersion of 3 : 4-benzpyrene containing 1 mg. per c.c. The animals were then killed at intervals of 1 to 4 hours depending upon the particular organ under investigation.

The duodenum and small intestine and the caecum and large intestine were subjected to an extraction procedure essentially that devised by Weigert and Mottram (1946) except that silica gel (100/200 mesh) was used in preference to silica (100/150 mesh) for chromatography. This modification was found to give a much higher development rate and cleaner products on elution with ethyl alcohol.

RESULTS

(a) *Gall bladder*

For examination of bile the animals were killed after 1 hour. The bright blue fluorescent bile was first extracted with ether but this removed only a small amount of fluorescent material. After acidification, however, an ether extract possessed a strong blue/white fluorescence and when examined spectroscopically was found to possess a fully aromatic benzpyrenoid type spectrum. It was obtained free from colouring matter by the method previously described for the 3-hydroxypyrene glucuronide (Harper, 1957), that is by evaporating a solution in slightly moist ether and washing the residue with several small volumes of benzene. The residue dissolved in ethyl alcohol possessed absorption maxima at 257, 267, 287, 298, 361, 379 and 412 mμ and in view of the absorption spectra obtained subsequently for purified X_1 and X_2 it was considered to contain a mixture of these two derivatives.

A solution of the mixture in water possessed an intense blue/white fluorescence which remained unchanged on the addition of alkali.

A sample, heated in dilute hydrochloric acid at 100° C. for 10 minutes, assumed a yellow colour and on extraction with benzene followed by chromatography on alumina yielded a bright yellow/green fluorescent zone near the surface and a reddish coloured, orange fluorescent zone moving slowly down the column. On elution with ethyl alcohol the yellow/green fluorescent zone yielded a blue fluorescent solution which spectroscopically appeared to be the F_1 of Weigert and Mottram. However, attenuation of the 395 and 418 mμ absorption bands suggested the presence of an additional substance. The orange fluorescent zone was similar in appearance and behaviour to synthetic benzpyrene-5 : 8-quinone but the small quantity available prevented complete characterisation.

A further sample of the mixture was incubated at 37° C. with β-glucuronidase (bacterial) at pH 7 for 2 hours. An ether extract of the incubated mixture was then colourless with a strong blue/white fluorescence. When examined spectro-

scopically it was found to possess the characteristic spectrum of F_1 but once again with attenuated 395 and 418 mμ absorption bands.

(b) *Duodenum and small intestine*

For examination of this tissue the animals were killed at 1½ to 2 hours. The final appearance of the chromatogram was essentially as described by Weigert and Mottram, namely a diffuse bright blue fluorescent zone extending downwards from the surface of the silica.

In one experiment the whole of this zone was eluted with ethyl alcohol, transferred to water and incubated at 37° C. with β-glucuronidase at pH 7 for 2 hours. An ether extract of the mixture then possessed the characteristic F_1 spectrum but with the same attenuated 395 and 418 mμ absorption bands

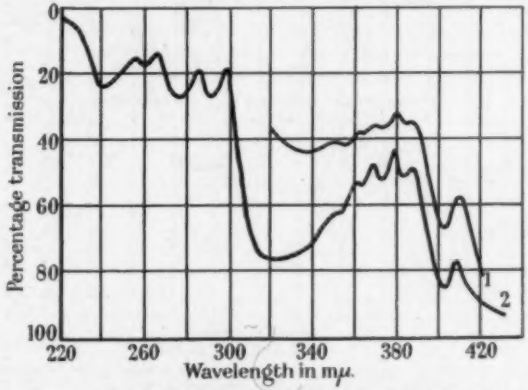


FIG. 1.—Absorption spectra in ethyl alcohol. 1. X_1 transcribed from Weigert and Mottram (1946). 2. X_1 as isolated in present work.

referred to above. On transferring to hexane these bands appeared as doublets with maxima respectively at 392 and 395–396 mμ and 415 and 419 mμ.

In a further experiment separation of the blue fluorescent zone on the silica gel into X_1 and X_2 was carried out by development with amyl alcohol as described by Weigert and Mottram.

X_1 metabolite.—The initial filtrate from the column was yellow in colour and non-fluorescent and accordingly was discarded. The remainder of the filtrate was diluted with 10 times its volume of petroleum ether (b.p. 40–60° C.) and the mixture rechromatographed on silica gel. The X_1 was obtained as a colourless, bright blue/white fluorescent zone beneath the surface. When eluted with ethyl alcohol and examined spectroscopically the X_1 was found to possess a fully aromatic benzpyrenoid type spectrum (Fig. 1) with maxima at 256, 266, 286, 298, 363, 369, 379, 388 and 408–409 mμ with an inflection at 352–354 mμ.

The purified X_1 was transferred to water and the following reactions observed.

(1) On heating in dilute hydrochloric acid at 100° C. for 10 minutes the X_1 was converted into pure F_1 and what appeared to be benzpyrene–5 : 8–quinone.

(2) On incubation at 37° C. with β-glucuronidase at pH7 for 2 hours it was converted almost quantitatively into pure F_1 (spectrum Fig. 2). Accordingly,

in view of its physical, chemical and biochemical behaviour allied with the new spectroscopic evidence which rules out the postulated diol type structure, it is concluded that the X_1 metabolite is the glucuronide conjugate of F_1.

X_2 metabolite.—After removal of X_1 from the silica gel chromatogram by prolonged development with amyl alcohol the whole of the column possessed a blue/white fluorescence with a greater intensity at the surface. The surface zone, when eluted with ethyl alcohol, yielded a yellow coloured solution possessing the spectrum shown in Fig. 2. Attempts to separate the X_2 from the apparent strongly absorbing background were unsuccessful but the indications are that X_2, like X_1, also possesses the fully aromatic benzpyrenoid configuration.

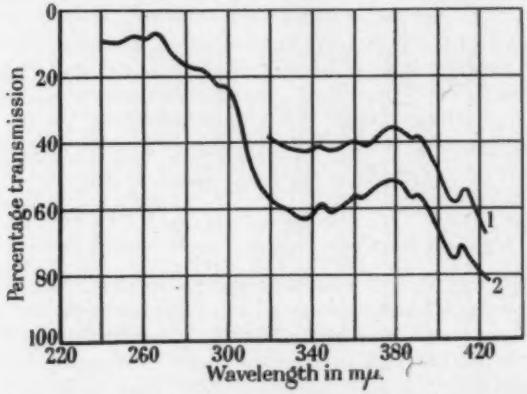


FIG. 2.—Absorption spectra in ethyl alcohol. 1. X_2 transcribed from Weigert and Mottram (1946). 2. X_2 as isolated in present work.

The crude X_2 thus obtained was incubated at 37° C. with β-glucuronidase at pH 7. After 2 hours a sample of the incubated mixture gave a fluorescence colour change from blue to green on addition of alkali. On extraction and chromatography on silica gel over alumina a small amount of unchanged X_2 was identified on the silica gel and a bright yellow/green fluorescent zone which appeared to be predominantly F_1 was obtained on the alumina.

It would appear therefore that X_2 is converted into F_1 and not F_2 by the enzymatic activity of β-glucuronidase. Unfortunately the possibility of contamination with X_1 cannot be ruled out, since, as was stated above, the whole of the silica gel possessed a blue/white fluorescence. Accordingly no definite conclusion can be drawn at present but it is perhaps significant that what was considered to be a purer sample of X_2 isolated from bile also yielded F_1 on enzymatic hydrolysis with β-glucuronidase.

(c) *Caecum and large intestine*

For examination of these organs the animals were killed after 4 hours. The extract was then found to contain F_1 as the major metabolite with only a trace amount of X derivatives. Once again however, the 395 and 418 mμ maxima of the F_1 were considerably attenuated and in hexane appeared as doublets with

maxima at 392 and 396 mμ and 415 and 419 mμ. The presence of 8-hydroxy-3 : 4-benzpyrene and possible trace of 5- or 10-hydroxy derivatives in the mixture was subsequently established by methylation and fluid chromatography.

The nature of the F_1 metabolite.—In distinction to the sky blue fluorescent appearance of F_1 adsorbed on alumina reported by Weigert and Mottram, throughout these experiments F_1 has appeared on the alumina chromatogram from benzene as a bright yellow/green fluorescent zone moving slowly down the column on development with solvent.

On addition of sodium hydroxide to a solution of F_1 in alcohol the fluorescence colour change from blue to yellow has occurred immediately and this behaviour was considered to be more in keeping with a free phenolic nature rather than the conjugated structure postulated by Weigert and Mottram.

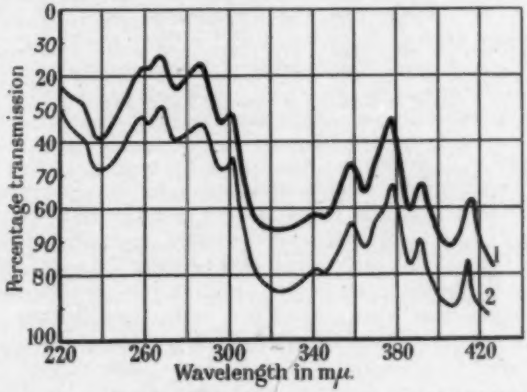


FIG. 3.—Absorption spectra in hexane. 1. F_1. 2. Methylated F_1.

Accordingly, in view of the conclusion arrived at by Berenblum and Schoental that F_1 is indistinguishable from unconjugated 10-hydroxy-3 : 4-benzpyrene, methylation of the pure F_1 obtained by hydrolysis of the purified X_1 with β-glucuronidase was carried out.

Methylation was effected with dimethyl sulphate and excess sodium hydroxide, the reaction being continued until the mixture no longer exhibited a yellow fluorescence in ultraviolet light. The mixture was then extracted with benzene and the benzene extract chromatographed on alumina. The methylated F_1 passed rapidly down the column and was collected in the filtrate. Further purification was effected by transference to cyclohexane followed by chromatography on alumina. The methylated derivative was obtained as a homogeneous blue/white fluorescent zone moving slowly down the column on prolonged development with solvent. It was eluted with ethyl alcohol and transferred to hexane for spectroscopic examination. The absorption spectrum (Fig. 3) is very similar to that of the F_1 with maxima at 259–260, 268, 287, 301, 342, 359, 378, 390 and 413 mμ. On comparison with the absorption spectra of the methylated metabolites of 3 : 4-benzpyrene previously identified in faeces, namely 8-benzpyrenol (Berenblum *et al.*, 1943), 10-benzpyrenol (Berenblum and Schoental, 1946) and 5-benzpyrenol (Pihar and Spálený, 1956) it is seen that the absorption spec

trum of methylated F_1 does not correspond to any of these (Table I). However, in view of the fact that F_1 readily undergoes oxidation to what appears to be the 5 : 8-quinone it seems likely that the hydroxyl group is at either the 5 or 8 position of the molecule. If this is so then the difference in spectral properties must be attributed to the presence of an additional group in the molecule.

TABLE I

(Transcribed in part from Pihar and Spálený, 1956)

3 : 4-Benzpyrene derivative (metabolic)	Source	Maxima in hexane—mμ									
F_1	—	261	268	–	287	301	342	358–359	378	392	415
Methylated F_1	—	259–260	268	—	287	301	342	359	378	390	413
8-Methoxy-	a	—	—	—	293	307	—	362	379	397	420
10-Methoxy-	b	257	267	278	287	297·5	347	—	374·5	395	411·5
5-methoxy-	c	256	266	274	287	299	—	357	375	395	409

a and b.—Berenblum and Schoental, 1946.
c.—Pihar and Spálený, 1956.

The F derivatives from the caecum and large intestine.—As was stated earlier spectroscopic examination of the F derivatives taken from the caecum and large intestine suggested that F_1 was the predominant component but that a small amount of an additional derivative was also present. Accordingly the mixture was methylated and chromatographed on alumina from cyclohexane as described above. The column was then developed with cyclohexane containing an increasing amount of benzene and the filtrate collected in fractions. The fractions were then transferred to alcohol and examined spectroscopically.

The spectrum of the first fraction was indeterminate but the presence of a distinct maximum at 408 mμ suggested the presence of a trace amount of either the 10-methoxy or the 5-methoxy derivative. Successive fractions were then identified as containing 8-methoxy-benzpyrene, mixtures of the 8-methoxy and methylated F_1 derivative and finally methylated F_1 alone.

It is concluded therefore that the attenuation of the 395 and 418 mμ absorption bands of F_1 in alcohol referred to previously is due largely to the presence of a small amount of 8-hydroxy-3 : 4-benzpyrene.

Attempted conversion of F_1 to F_2

As evidence for their postulated conjugated structure for F_1 Weigert and Mottram stated that F_1 was converted into 8-hydroxy-3 : 4-benzpyrene by prolonged heating in sulphuric acid.

Attempts to repeat this transformation have proved unsuccessful. Thus on heating with 0·5 N sulphuric acid in the presence of air the F_1 was rapidly converted to a non-fluorescent compound which appeared on an alumina chromatogram as a red zone with an orange fluorescence similar to that of synthetic 5 : 8-quinone. Owing to the small amount of derivative involved however, attempts to effect further characterisation by reductive methylation were not successful. When the heating was carried out under nitrogen for as long a period as 8 hours the F_1 was recovered unchanged.

Attempts to convert F_1 into F_2 by enzymatic activity proved equally unsuccessful. Thus F_1 was recovered unchanged after incubation with β-glucuronidase or with an homogenate of caecum and large intestine taken from untreated mice.

DISCUSSION

Contrary to the assertion of Weigert and Mottram that in mixtures of F_1 and F_2 in alcohol the last two bands in the absorption spectrum appear as doublets this phenomenon has not been observed during the present experiments. It is concluded from the methylation experiments carried out on the F derivatives isolated from the caecum and large intestine that the effect of a small amount of 8-hydroxy-3 : 4-benzpyrene on a solution of F_1 in alcohol is merely to attenuate the 395 and 418 mμ absorption bands of the F_1.

Accordingly it is suggested by these experiments that, during the first hour following intravenous injection of benzpyrene, F_1 and F_2 are eliminated in the bile as glucuronide conjugates, that of F_1 being considerably in excess of the F_2 conjugate. This mixture then passes unchanged through the duodenum and small intestine and is hydrolysed to the phenols F_1 and F_2 during subsequent passage through the caecum and large intestine. The findings of Marsh, Alexander and Levvy (1952) with respect to the intestinal distribution of glucuronide decomposing enzymes, referred to in the preceding communication on the intermediary metabolism of pyrene, once again provide important circumstantial evidence in support of this view.

From the physical, chemical and biochemical properties of X_1 cited above the glucuronide conjugate of F_1 is considered to be X_1. It is tempting to conclude therefore that X_2 is a similar conjugate of F_2 but the experimental evidence does not support this view. No positive conclusion can be drawn at present however, owing to the impurity of the X_2 obtained and the possible contamination with X_1 metabolite.

It appears therefore that this metabolic sequence presents an analogous case to that found for the non-carcinogenic pyrene. The major, and perhaps important, difference, lies in the presence of two distinct metabolic pathways in the case of 3 : 4-benzpyrene. Further experiments are to be carried out with other hydrocarbons to determine whether there is an association between an alternative metabolic mechanism and the carcinogenic process.

SUMMARY

(1) The intermediary metabolite X_1 of 3 : 4-benzpyrene has been found to possess the fully aromatic benzpyrenoid configuration and its physical, chemical and biochemical properties are consistent with it being the glucuronide conjugate of F_1.

(2) The experimental evidence suggests that F_2 is also present in the bile and small intestine as a glucuronide conjugate, but the evidence is not consistent with this conjugate being the X_2 metabolite. However, owing to the impure nature of the X_2 obtained no definite conclusion can be drawn at present.

(3) The full absorption spectrum of the end product F_1 has been recorded and is consistent with a fully aromatic benzpyrenoid configuration for this compound.

(4) F_1 on methylation has yielded a derivative which differs spectroscopically from the methylated derivatives of the 8-, 10- and 5-benzpyrenols previously identified in faeces.

(5) The metabolism of 3 : 4-benzpyrene is discussed in relation to that of the non-carcinogenic pyrene.

REFERENCES

BERENBLUM, I., CROWFOOT, D., HOLIDAY, E. R. AND SCHOENTAL, R.—(1943) *Cancer Res.*, **3**, 151.
Idem AND SCHOENTAL, R.—(1946) *Ibid.*, **6**, 699.—(1955) *Science*, **122**, 470.
CHALMERS, J. G.—(1938) *Biochem. J.*, **32**, 271.
Idem AND CROWFOOT, D.—(1941) *Ibid.*, **35**, 1270.
Idem AND PEACOCK, P. R.—(1936) *Ibid.*, **30**, 1242.
HARPER, K. H.—(1958) *Brit. J. Cancer*, **12**, 116.
MARSH, C. A., ALEXANDER, F. AND LEVVY, G. A.—(1952) *Nature*, **170**, 163.
PIHAR, O. AND SPÁLENY, J.—(1956) *Chem. Listy.*, **50**, 296.
WEIGERT, F. AND MOTTRAM, J. C.—(1946) *Cancer Res.*, **6**, 97.

ADDENDUM

Since this paper was submitted for publication the author's attention has been drawn to the suggestion by Chalmers (1956) that 3 : 4-benzpyrene is metabolised in the liver to hydroxy derivatives which are conjugated with glucuronic acid. The metabolites are then excreted either in the urine or in the faeces after hydrolysis in the gut.

In a more recent communication Conney, Miller and Miller (1957) have reported that 3 : 4-benzpyrene is metabolised by fortified rat liver homogenate to a mixture of the 8- and 10-benzpyrenols and an unidentified compound corresponding to the F_1 of Weigert and Mottram together with small amounts of 5 : 8- and 5 : 10-quinones and 5 : 8-dihydroxy-3 : 4-benzpyrene. The properties of the F_1 resembled those of a monohydroxy-benzpyrene and on methylation yielded a derivative with absorption maxima at 412, 389, 377, 358, 299, 285, 258, and 240 mμ. In the longer wave band region these are in close agreement with the absorption maxima obtained for methylated F_1 during the present experiments.

They also report, however, that their F_1 band, upon methylation, yielded appreciable amounts of 8-methoxy-benzpyrene in addition to the methylated F_1. In the present experiments a similar mixture was obtained upon methylation of the F_1 zone isolated from the large intestine and caecum, but a pure sample of F_1, obtained by β-glucuronidase hydrolysis of the X_1 metabolite, yielded only methylated F_1.

REFERENCES

CHALMERS, J. G.—(1956) *Rep. Brit. Emp. Cancer Campgn.*, **34**, 286.
CONNEY, A. H., MILLER, E. C. AND MILLER, J. A.—(1957) *J. biol. Chem.*, **228**, 753.

THE CULTIVATION *IN VITRO* OF MOUSE ASCITES TUMOUR CELLS. THE EFFECTS OF MONOCYTES ON THE GROWTH OF THE TUMOUR CELLS.

A. K. POWELL

From the Department of Experimental Pathology, Mount Vernon Hospital, Northwood, Middlesex

Received for publication January 29, 1958

It was reported earlier (Powell, 1957*d*) that the growth *in vitro* of Ehrlich carcinoma and Sarcoma 37 ascites tumour cells was promoted by the presence of explants of various tissues. These explants were histologically heterogeneous so it was not possible to identify the particular types of cells which produced the soluble growth-promoting factors. However, monocytes derived from adult mouse tissues were implicated as possible sources of these factors. It was also reported (Powell, 1957*c*) that macrophages from ascitic tumour fluid protected individual ascites tumour cells from the harmful effects of relative isolation.

As monocytes could be readily obtained in almost "pure culture", studies of the growth-promoting effects of these cells upon ascites tumour cells were made.

MATERIALS AND METHODS

Mice and tumours.—Ehrlich carcinoma and Sarcoma 37 ascites tumours were maintained in RIII strain mice as described previously (Powell, 1957*a*).

Tissue cultures.—Monocytes were cultured from fragments of spleen, taken from adult RIII strain mice, by the double-coverslip method. The general methods used, including the preparation of media components, were similar to those employed earlier (Powell, 1957*a*).

The spleen explants were cultivated in fluid media consisting of 40 per cent Earle's buffered saline solution, 20 per cent of 1 : 4 chick embryo extract, and 40 per cent of either heparinized ascites tumour plasma or serum. The serum was obtained from plasma incubated overnight without added heparin. The proportion of centrifuged 11-day chick embryo tissue was about one quarter of that of the supernatant Earle's solution extract. Cultures were incubated in the lying-drop position to avoid unnecessary loss of monocytes. In this position the migrating cells adhered to the coverslips.

Small wandering cells rapidly migrated from the explants. They were later followed by increasing numbers of monocytes. During subculturing, carried out at intervals of 48 hours, most of the small amoebocytes were washed off coverslips bearing serum medium when these were bathed in Earle's solution prior to renewal of the culture medium. These cells tended to adhere more firmly to the protein film on the coverslips with plasma medium until liquefaction ensued. Six days after setting up the cultures the explants had visibly decreased in size. This

was associated with the great output of cells. At this time the explants seemed to consist mainly of massed monocytes. Most of the small amoebocytes had been removed and a large proportion of those left on the coverslips were degenerated. Although some monocytes had been lost during subculturing those remaining were abundant and healthy (Fig. 1) and attached to the coverslips.

The experimental double-coverslip cultures were set up about seven days after explantation of the spleen fragments. Combined cultures of monocytes with each strain of ascites tumour cells, cultures of monocytes alone, and " pure cultures " of tumour cells were prepared. The combined cultures differed in the numbers and arrangements of the monocytes. In some the explants were placed on fresh coverslips and in others the parent coverslips bearing only numerous migratory monocytes were used. In a third group the original coverslips with explants and monocytes were retained.

Selected ascites tumour fluid containing actively dividing and healthy tumour cells was serially diluted with a mixture of equal parts of fowl and ascites tumour plasma to give a range of dilutions and densities of tumour cell population. To prepare the combined cultures equal volumes of the cell suspension and dilute chick embryo extract were mixed and this inoculum quickly and evenly spread over the coverslips bearing the explants and/or monocytes. In each instance " pure cultures " of tumour cells were prepared from the same batches of inoculum used for the combined cultures. The fates of the tumour cells in the combined and collateral " pure cultures " were compared. The cultures were washed in Earle's solution and supplied with nutrient medium at intervals of 48 hours. This medium consisted of 40 per cent horse serum and 60 per cent Earle's solution with a small proportion of embryo extract. Cultures were fixed in " Susa " mixture at daily intervals and stained with dilute Harris' haematoxylin.

EXPERIMENTAL RESULTS

The " pure cultures " of ascites tumour cells behaved as described previously (Powell, 1957*a*, *b*). In thinly populated cultures the great majority of the tumour cells degenerated during the first 24 hours of incubation. In cultures with a medium density of population most of the tumour cells were healthy after cultivation for 24 hours but thereafter degenerated. Comparison of " pure cultures " with collateral combined cultures showed that monocytes protected the tumour cells of both strains from the harmful effects of relative isolation. This protection was clearest in cultures with widely separated tumour cells.

The results of the present experiments were similar to those obtained with explants of spleen, thymus and lymph node (Powell, 1957*d*) and are therefore only briefly discussed. However, unlike fresh explants, the subcultured fragments of spleen in experimental cultures did not liberate relatively large amounts of protective factors during the first 24 hours of cultivation. Thus the protective effects of the subcultured spleen explants extended for only a short distance in 24-hour-old cultures with widely dispersed tumour cells. This distance was comparable to that previously found in similar 48-hour-old cultures which had been prepared with fresh spleen explants.

After 24 hours the incidence of degenerated tumour cells in cultures with medium densities of population increased, especially away from the explants. As the combined cultures aged the incidence of degenerated tumour cells increased.

The explants themselves slowly degenerated. This was perhaps due partly to the retention of the original plasma coagulum and to loss of monocytes in saline baths during subculturing. The parallel between the present and the earlier experiments, when fresh explants were used, was close. Both viability and division of the tumour cells were primarily determined by their distance from groups of free monocytes or explants. Viable resting cells extended further than dividing cells from the explants.

On the 6th day of cultivation living tumour cells were confined to the close neighbourhood of the explants and groups of monocytes (Fig. 2, 3) ; some of these tumour cells were found to be dividing (Fig. 3). The tumour cells were easily distinguished from the monocytes by their morphology and greater basophilia as shown in the photomicrographs. The protective effects of free monocytes were limited to their immediate vicinity. They were much weaker than those of the explants themselves. This was probably a quantitative difference as the explants were composed essentially of massed monocytes. Tumour cells of both strains were predominantly epithelioid. Aggregations of tumour cells, apparently formed by coherence of daughter cells, were common in older cultures.

Spleen monocytes very considerably prolonged the survival and duration of the ability to reproduce of the ascites tumour cells. This protective effect was mediated by substances diffusing through the coagulum since the monocytes were not necessarily in contact with the tumour cells.

DISCUSSION

Ascites tumour cells of the strains studied were unable to grow progressively when maintained in " pure culture " in plasma media (Powell, 1957*a*, *b*). Cultivated in the presence of explants of various tissues (Powell, 1957*d*) or of monocytes, as described above, the tumour cells remained healthy and divided so long as the explants and normal cells were themselves active. The protection given by the explants and/or amoebocytes has been ascribed to their production of protective and growth-promoting substances which diffused through the culture medium to the often widely scattered tumour cells.

The interactions of normal and neoplastic cells cultivated together *in vitro* have been studied by many workers. Reciprocal stimulation of Ehrlich carcinoma cells of solid explants and fibroblasts was reported by Fischer *et al.* (1929). Ludford and Barlow (1942, 1945) found that the growth of fibrocytes was stimulated by carcinoma but inhibited by sarcoma cells. These interactions were mediated by the culture medium. These workers (1945) suggested that stimulation of fibrocyte growth by mammary carcinomas was implicated in the development of sarcomas derived from them. Sanford *et al.* (1951) obtained sarcomas at the sites of inoculation into mice of cultures of hepatoma, melanoma and thyroid cultures after prolonged cultivation *in vitro*. They appear to have believed that fibroblasts underwent a malignant transformation during serial cultivation.

Maximow (1925) observed a " morphologically malignant " transformation of cultivated normal rabbit mammary gland tissue. The changed epithelial cells invaded and destroyed connective tissue cells *in vitro*. Santesson (1935) reported that malignant mammary tumour cells diffusely infiltrated connective tissue cells in culture but that benign tumour cells were often encapsulated by the normal cells. Mouse lymphosarcoma (T86157 MB) cells were originally dependent

upon the presence of fibrocytes for growth *in vitro* (De Bruyn, Korteweg and van Waveren, 1949) but later (De Bruyn and Gey, 1952) altered to a cell type then independent of fibrocytes. Bichel (1952) reported that normal tissue was required for the growth of and destroyed by cultivated leukaemic cells. Leighton and Kline (1954) observed that HeLa cells infiltrated and destroyed connective tissue in sponge matrix cultures ; in the absence of connective tissue the growth of the carcinoma cells was insignificant.

Carrel (1925) reported that bone marrow macrophages greatly stimulated the growth of Crocker rat Sarcoma 10 *in vitro*. Carrel (1922, 1924*a*) had previously shown that extracts of leucocytes also greatly stimulated the growth of fibroblasts. Ludford (1940) found that buffy coat leucocytes—probably monocytes and macrophages—without being in contact with them promoted the growth of fibroblasts and sarcoma cells.

Schleich (1954) observed that free amoeboid tumour cells from explants of solid tumours produced by subcutaneous injection of ascites tumour fluid were associated with fibrocytes. In a study of the growth *in vitro* of explants of subcutaneous Yoshida rat sarcoma produced by inoculation with ascites fluid, Schleich (1956) reported that individual tumour cells failed to grow in the absence of fibrocytes. She believed that the tumour cells were unable to use the medium directly and depended on a metabolic product of the fibroblasts.

Such evidence collectively suggests that neoplastic cells benefit from the presence of normal cells, even to being completely dependent upon them in certain conditions, and that the fates of normal cells in mixed cultures are related to the grades of malignancy and histological natures of the tumour cells. On the other hand normal cells of different types cultivated together appear to interact without adverse effects.

Thus Ebeling and Fischer (1922) found that a " pure strain " of iris epithelium grown in mixed cultures with a " pure strain " of heart fibroblasts developed a glandular arrangement of polarized cells. Organ-like complexes of an outer epithelial layer surrounding mesenchymatous tissue were cultured from embryonic intestine by Fischer (1922). Embryonic chick cartilage was reported by Fischer (1931) to grow *in vitro* in the presence of either perichondral cells or heart fibroblasts but not alone. New cartilage was apparently formed by transformation of fibroblasts in the presence of living cartilage. A peculiar amalgamated tissue developed in mixed cultures of osteoblasts and heart fibroblasts (Fischer, 1946*b*). Willmer (1954*b*) stated that the presence of fibrocytes promotes the differentiation of epithelial cells ; the fibrocytes might produce a substance necessary to the epithelial cells.

The failure of Ehrlich carcinoma ascites tumour cells to grow in fluid or plasma media was ascribed by Barka *et al.* (1955) to inability to proliferate when isolated. These authors assumed that the tumour cells needed the presence of normal cells for multiplication and that a process of " enzymatic cooperation " rather than a " simple interconnection between substances " was involved. Powell (1957*a*) inferred from the spatial arrangement of the ascites tumour cells in plasma cultures, together with observed correlations between duration of viability and incidence of cell division, respectively, and density of tumour cell population that the inability of the cells to grow was probably due to their failure to form adequate amounts of essential soluble substances. When cultivated in fine-bore glass capillaries (Powell, 1957*c*) at low population densities the tumour cells in " pure

culture " survived and divided for longer periods than they did in open coverslip cultures. This difference was attributed to the presence of higher concentrations of the diffusible factors within the limited volumes of the capillaries.

The failure of isolated ascites tumour cells to grow *in vitro* appears to be due to nutritive inadequacy of the medium employed. This deficiency has been experimentally remedied by the presence of living cells of other types. A difference in nutritive requirements between normal and neoplastic cells might be important. Researches on the cultivation of single cells are relevant to this possibility.

Early attempts to cultivate single cells were unsuccessful (Fischer, 1923, 1946*a*; Barnard, 1925). Moen (1935) reported that division of relatively isolated fibrocytes *in vitro* was promoted by close proximity of other fibrocytes and succeeded in obtaining clones from such cells. The successful establishment of a clone from a single cell culture was achieved by Sanford, Earle and Likely (1948); L strain cells were found to divide when confined in small volumes of medium previously conditioned by living cells of the same strain. Likely, Sanford and Earle (1952) later obtained clones from a variety of cells in both conditioned and unconditioned media. Earle *et al.* (1951) showed that a certain minimum population density of cell was necessary to ensure survival and that larger numbers were needed for multiplication. The importance of cell population in relation to cell activity has been discussed by Earle, Bryant and Schilling (1954).

Puck and Marcus (1955) described the production of clones of HeLa cells by using non-dividing irradiated feeder cells as a source of protective factors. Failure of isolated HeLa cells to divide in the absence of feeder cells was attributed to loss of short-lived diffusible substances from the cells. Puck, Marcus and Cieciura (1955) then successfully cultured single HeLa cells, isolated by a less injurious method from stock cultures, in the absence of feeder cells. Clones were also grown from other types of cells. These positive results of Puck and his colleagues may have been due in part to the culture medium used. Leighton and Kline (1954) had found with a different medium that HeLa cells required the presence of fibrocytes for good growth. Similarly, Puck and Fisher (1956) isolated as clones two contrasting types of HeLa cells (S1 and S3), only one of which (S1) required feeder layers for single cell proliferation in the complete medium. It was then shown that a feeder layer of S3 cells enable single S3 cells to overcome an inositol deficiency of incomplete medium (Fisher and Puck, 1956). The failure of isolated cells to grow is presumably due, in the absence of trauma, to unsuitability of the medium used (Sanford *et al.*, 1948; Earle *et al.*, 1954; Fisher and Puck, 1956).

The nutritive substances absent in plasma/embryo extract media, quantitatively if not qualitatively, needed by ascites tumour cells have been shown to be produced by amoebocytes. The diffusible substances produced by leucocytes which promoted the growth of fibrocytes *in vitro* were termed " trephones " by Carrel (1922, 1924*b*). Saline extracts of brain, thymus, spleen and bone marrow, but not of liver, kidney or muscle were found to be growth-promoting by Trowell and Willmer (1939). Explants of embryonic liver, kidney and muscle, in contrast to those of the corresponding adult tissues (unpublished data), have been found to be strongly protective and growth-promoting to dispersed ascites tumour cells.

The active substances in potent adult tissue, embryo tissue and amoebocytes are comparable. They are soluble in saline solutions, labile and growth-promoting.

Amoebocytes do not require embryo extract for growth and division (Carrel and Ebeling, 1926). They grow and divide in serum diluted with Tyrode's solution (Baker, 1933). Monocytes divide some 20 hours after application of fresh serum medium (Jacoby, 1938). Fibrocytes, on the other hand, require embryo extract for proliferation; divisions begin about 10 hours after the provision of embryo extract (Willmer and Jacoby, 1936; Jacoby, Trowell and Willner, 1937). It was also shown by Jacoby *et al.* (1937) that the stimulatory effects of embryo extract on fibrocyte growth were initiated within a relatively short time after its application. These differences between amoebocytes and fibrocytes suggest that there is a rapid utilization of preformed stimulating substances by fibrocytes and a synthesis of such substances followed by utilization by amoebocytes. It is possible that amoebocytes are a primary source of growth-promoting factors in the organism.

Active growth *in vitro* of explants of fresh solid tumour is associated with large numbers of wandering cells. Earle (1937) pointed out that Walker rat Carcinoma cells infiltrated muscle fragments *in vitro* only slowly in comparison with the rapid infiltration seen *in vivo*. Ludford (1940) suggested that this difference was perhaps due to the presence of monocytes and macrophages in the tumour *in vivo* as these are relatively few in such cultures. Willmer (1954*a*) suggested that the continued growth of fibrocyte cultures may depend on the usual slight "contamination" with amoebocytes. The protective effects upon scattered ascites tumour cells of various explants (Powell, 1957*d*) may be closely associated with amoebocytes present in them.

The nutritive needs of strains of cells as HeLa carcinoma (Gey, Coffman and Kubicek, 1952) and L strain sarcomatous fibroblasts (Sanford *et al.*, 1948) which have been serially cultivated for prolonged periods may differ appreciably from those of freshly explanted tumour cells. Their requirements may be less stringent in view of their exposure to selection for growth under *in vitro* conditions. For example, T 86157 MB lymphosarcoma cells became more self-sufficient with continued culture (De Bruyn and Gey, 1952). Puck and Fisher (1956) demonstrated the existence in HeLa stock cultures of cell lineages with different nutritive requirements. The neoplastic cells of tumours, and especially spontaneous ones, may be less autonomous and more vulnerable.

In contrast with explant cultures and "pure cultures" derived by repeated subculturing, the establishment of clones of malignant and normal cells (Likely, Sanford and Earle, 1952; Marcus, Cieciura and Puck, 1956) has shown that heterogeneous cells populations and leucocytic trephones are not always necessary for the growth of cells *in vitro*. However, this appears to depend on the use of nutritionally complete or conditioned medium for cultivation of single cells, or the presence of a certain minimal number of cells in the more usual media.

Survival and growth of Ehrlich carcinoma and Sarcoma 37 ascites tumour cells cultivated in plasma-embryo extract media appear to depend upon trephone-like substances which they do not themselves produce in sufficient amounts. Such substances are released from co-existent normal cells and may be initially synthesized by amoebocytes. This inter-cellular dependence may be peculiar to ascites tumour cells but in view of the present conclusions a comparative study of the possible dependence in some conditions of neoplastic and normal cells upon other cells, especially amoebocytes, and their products may be relevant to the biological properties of tumour cells in general.

SUMMARY

The protective and growth-promoting effects of spleen monocytes on Ehrlich carcinoma and Sarcoma 37 ascites tumour cells cultivated in plasma media are described.

Interactions between normal and neoplastic cells cultivated *in vitro* are discussed.

It is concluded that the growth-promoting effects of monocytes and protective explants are due to the liberation from them of trephone-like substances into the culture medium.

These substances compensate for the nutritive inadequacy of the plasma-embryo media used for the cultivation of ascites tumour cells.

I am indebted for assistance with the *in vitro* research to Mr. G. A. Butcher and with the maintenance of the tumours *in vivo* to Mr. F. Butcher.

The expenses of this work were defrayed from a block grant by the British Empire Cancer Campaign.

REFERENCES

Baker, L. E.—(1933) *J. exp. Med.*, **58**, 575.
Barka, T., Töro, I., Posalaky, Z. and Rappay, G. Y.—(1955) *Acta Morph. Acad. Sci. Hung.*, **6**, 87.
Barnard, J. E.—(1925) *Brit. J. exp. Path.*, **6**, 39.
Bichel, J.—(1952) *Acta path. microbiol. scand.*, **31**, 40.
Carrel, A.—(1922) *J. exp. Med.*, **36**, 385.—(1924*a*) *C.R. Soc. Biol. Paris*, **90**, 29.—(1924*b*) *J. Amer. med. Ass.*, **82**, 255.—(1927) *C.R. Soc. Biol. Paris*, **97**, 19.
Idem and Ebeling, A. H.—(1926) *J. exp. Med.*, **44**, 261, 285.
De Bruyn, W. M. and Gey, G. O.—(1952) *Acta Un. int. Cancr.*, **7**, 772.
Idem, Korteweg, R. and van Waveren, E. K.—(1949) *Cancer Res.*, **9**, 282.
Earle, W. R.—(1937) *Arch. exp. Zellforsch.*, **20**, 140.
Idem, Bryant, J. C. and Schilling, E. L.—(1954) *Ann. N.Y. Acad. Sci.*, **59**, 1000.
Idem, Sanford, K. K., Evans, V. J., Waltz, H. K. and Shannon, J. E.—(1951) *J. nat. Cancer Inst.*, **12**, 133.
Ebeling, A. H. and Fischer, A.—(1922) *J. exp. Med.*, **36**, 285.
Fischer, A.—(1922) *Ibid.*, **36**, 393.—(1923) *Ibid.*, **38**, 667.—(1931) *Arch. EntwMech. Org.*, **125**, 203.—(1946*a*) 'Biology of Tissue Cells'. London (Cambridge University Press), pp. 16, 171.—(1946*b*) *Ibid.*, p. 226.
Idem, Laser, H. and Meyer, H.—(1929) *Z. Krebsforsch.*, **29**, 270.
Fisher, H. W. and Puck, T. T.—(1956) *Proc. nat. Acad. Sci.*, **42**, 900.
Gey, G. O., Coffman, W. D. and Kubicek, M. T.—(1952) *Cancer Res.*, **12**, 264.
Jacoby, F.—(1938) *J. Physiol.*, **93**, 48P.
Idem, Trowell, O. A. and Willmer, E. N.—(1937) *J. exp. Biol.*, **14**, 255.
Leighton, J. and Kline, I.—(1954) *Tex. Rep. Biol. Med.*, **12**, 865.
Likely, G. D., Sanford, K. K. and Earle, W. R.—(1952) *J. nat. Cancer Inst.*, **13**, 177.
Ludford, R. J.—(1940) *Brit. Med. J.*, i, 201.
Idem and Barlow, H.—(1942) *Cancer Res.*, **4**, 694.—(1945) *Ibid.*, **5**, 257.
Marcus, P. I., Cieciura, S. J. and Puck, T. T.—(1956) *J. exp. Med.*, **104**, 615.
Maximow, A.—(1925) *Contr. Embryol., Carneg. Instn., Pub.* 16. No. 80, 47.
Moen, J. K.—(1935) *J. exp. Med.*, **61**, 247.
Powell, A. K.—(1957*a*) *Brit. J. Cancer*, **11**, 274.—(1957*b*) *Ibid.*, **11**, 280.—(1957*c*) *Ibid.*, **11**, 478.—(1957*d*) *Ibid.*, **11**, 570.

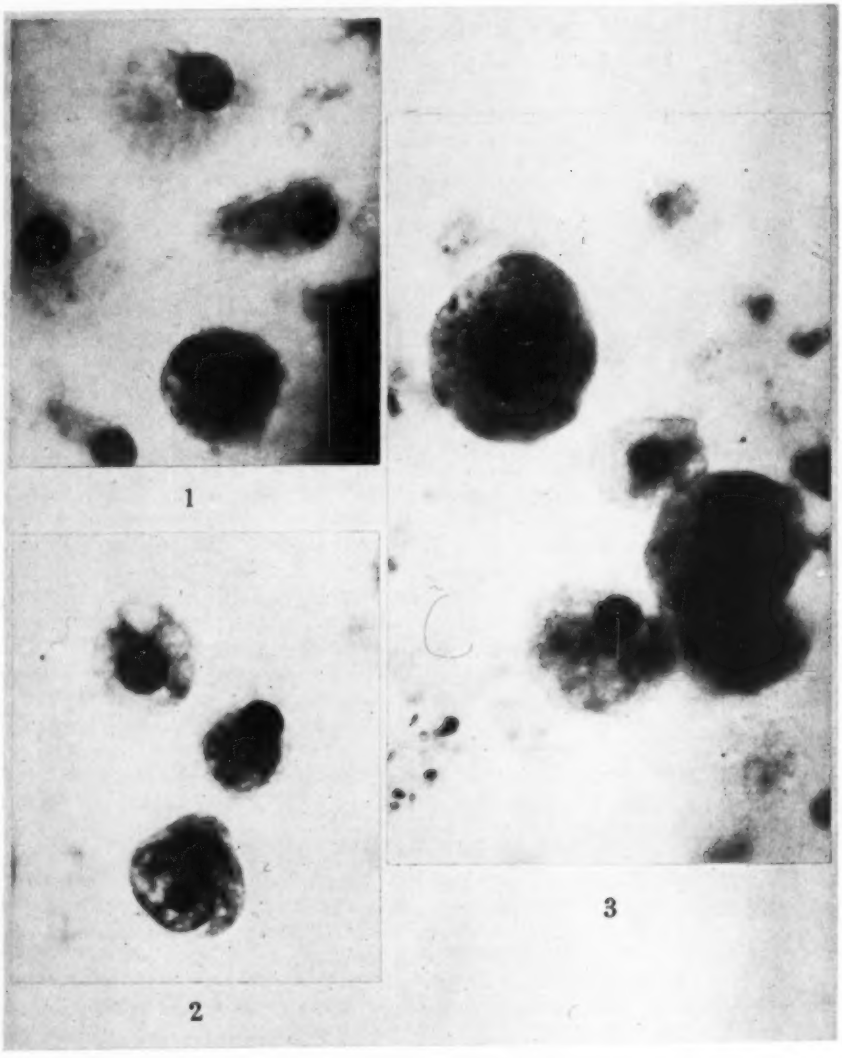
PUCK, T. T. AND FISHER, H. W.—(1956) *J. exp. Med.*, **104**, 427.
Idem AND MARCUS, P. I.—(1955) *Proc. nat. Acad. Sci.*, **41**, 432.
Iidem AND CIECIURA, S. J.—(1955) *J. exp. Med.*, **103**, 273.
SANFORD, K. K., EARLE, W. R. AND LIKELY, G. D.—(1948) *J. nat. Cancer Inst.*, **9**, 229.
Idem, LIKELY, G. D., EVANS, V. J., MACKEY, C. J. AND EARLE, W. R.—(1951) *Ibid.*, **12**, 1057.
SANTESSON, L.—(1935) *Acta path microbiol., scand., Suppl.*, **24**, 1.
SCHLEICH, A.—(1954) *Cancer Res.*, **14**, 486.—(1956) *Ann. N.Y. Acad. Sci.*, **63**, 849.
TROWELL, O. A. AND WILLMER, E. N.—(1939) *J. exp. Biol.*, **16**, 60.
WILLMER, E. N.—(1954*a*) 'Tissue Culture'. London (Methuen: Biological Monographs), p. 95.—(1954*b*) *Ibid.*, p. 130.
Idem AND JACOBY, F.—(1936) *J. exp. Biol.*, **13**, 237.

EXPLANATION OF PLATE

FIG. 1.—Monocytes attached to surface of coverslip. Culture of spleen explant incubated for 6 days.

FIG. 2.—Viable Ehrlich carcinoma ascites tumour cell adjacent to spleen monocytes. Experimental culture incubated for 6 days.

FIG. 3.—Dividing Sarcoma 37 ascites tumour cells adjacent to spleen monocytes. Experimental culture incubated for 6 days.



1

2

3

SOME EFFECTS OF NITROGEN AND SULPHUR MUSTARDS ON THE METABOLISM OF NUCLEIC ACIDS IN MAMMALIAN CELLS

R. B. DRYSDALE, AGNES HOPKINS, R. Y. THOMSON, R. M. S. SMELLIE AND J. N. DAVIDSON

From the Department of Biochemistry, the University of Glasgow

Received for publication November 11, 1957

OBSERVATIONS from a number of different fields suggest that di-(2-chloroethyl) sulphide (sulphur mustard, H) and methyl-di-(2-chloroethyl)amine (nitrogen mustard, HN2) have a specific effect on the process of nuclear division (Philips, 1950). In view of the importance of deoxyribonucleic acid (DNA) in cell division, it might be expected that sulphur and nitrogen mustards would exert some specific effect on its metabolism.

A clear demonstration of such an effect of sulphur mustard on DNA synthesis has been given by Herriott (1950–51) who showed that the addition of sulphur mustard to growing cultures of *E. coli B.* resulted in a cessation of DNA synthesis while ribonucleic acid (RNA) synthesis continued at 65–75 per cent of the normal rate. Bodenstein and Kondritzer (1948), who exposed salamander embryos to a solution of nitrogen mustard, demonstrated that the RNA concentration continued to increase in the normal manner while the DNA concentration remained at the level reached at the time of exposure. The effect of HN2 on the incorporation of (^{14}C)formate and (1 : 3–^{15}N)adenine into the nucleic acids of the small intestine of the rat was investigated by Goldthwait (1952), who found that, whereas HN2 produced a marked decrease of isotope incorporation into DNA, little or no change was produced in the isotope content of RNA.

The present experiments were undertaken to determine whether in mammalian tissues nitrogen and sulphur mustards exert a specific effect on DNA synthesis.

EXPERIMENTAL

Mustard.—Sulphur mustard (supplied by The Ministry of Supply, C.D.E.E., Porton, Wiltshire) was made up as a 5 per cent (w/v) solution in glyceryl triacetate for *in vivo* experiments and in horse serum (125 μg./ml.) for *in vitro* experiments. Nitrogen mustard (Mustine hydrochloride B.P.C., Boots Pure Drug Co.) was dissolved in 0·9 per cent (w/v) NaCl for *in vivo* and some *in vitro* experiments and in distilled water for other *in vitro* experiments. In all cases solutions of HN2 were prepared immediately before use.

Animals.—Male albino rabbits weighing from 1500–2000 g. were used. H or HN2 was injected into the marginal vein of the left ear. After a period of from 2 to 96 hours 1 mc. of carrier-free $^{32}PO_4^{\equiv}$ was injected intramuscularly into the thigh and two hours later the animal was killed by cervical dislocation. The tissues were excised and chilled in ice; those not worked up immediately were stored at −20° C. Just prior to killing 5 ml. of blood were removed from

the marginal vein of the right ear and used for measurement of the blood inorganic phosphate activity as described by Smellie, Humphrey, Kay and Davidson (1955).

Ascites cells.—The Ehrlich ascites tumour was maintained in ordinary stock mice from the Departmental colony. The MClM tumour was maintained in C3H F_1 hybrids (Klein, 1951).

For each experiment isotope was added to the pooled ascitic fluid from several mice, 2 to 4 ml. portions were dispensed into roller tubes containing the appropriate additions and the tubes were incubated in air on a roller drum at 37° C. In some cases the cells were separated from the ascitic plasma by centrifuging, resuspended in Krebs-Ringer bicarbonate (Cohen, 1951) and washed by low speed centrifuging. This washing was repeated 3 or 4 times until most of the blood cells had been removed. The tumour cells were then resuspended in Krebs-Ringer bicarbonate, isotope was added and portions of 5 or 10 ml. were incubated with shaking at 37° C. in 50 ml. Erlenmeyer flasks containing the appropriate additions. After incubation the vessels were frozen in dry ice and alcohol and kept at −20° C. until required for processing.

Tissue cultures.—Cells of Earle's "L" strain (Sanford, Earle and Likely, 1948) were used. Batches of replicate cultures of about 2×10^6 cells were set up in 50 ml. Erlenmeyer flasks and allowed to grow for 18–24 hours. The flasks were divided randomly into groups of 4 and nitrogen mustard in Hanks' Balanced Salt Solution (Hanks and Wallace, 1949) added. When the cells had been exposed to HN2 for 4 hours the fluid phase was replaced by 6 ml. fresh medium and about 40 hours later the medium was again renewed. After a further interval of 48 hours the medium was decanted, the cells extracted with lipid solvents and either stored at −20° C. or processed immediately.

Liver extract was prepared from mouse liver as described by Schulman, Sonne and Buchanan (1952).

Analytical methods.—For processing and analysing the material from experiments on ^{32}P incorporation the methods of Smellie *et al.* (1955) were used and for experiments on ^{14}C incorporation the procedure of Smellie, Thomson and Davidson (1958) was adopted. In both cases a preliminary fractionation of nuclear and cytoplasmic material was carried out as described by Smellie *et al.* (1955) in certain experiments in order to separate nuclear RNA (nRNA) from cytoplasmic RNA (cRNA). For the tissue culture experiments the methods described by Paul (1957) were employed.

RESULTS

Tables I and II show the effects of H and HN2 on the incorporation of $^{32}PO_4^{\equiv}$ into the nucleic acids of rabbit tissues *in vivo*. At a dose level of 1·5 mg./kg., H produces a marked depression of incorporation into appendix DNA after 2 hours; this depression is accentuated after 24 hours and is still apparent after 48 hours. The effects on appendix nRNA and cRNA are much less dramatic; only at 24 hours is a significant depression evident. Similarly only at 24 hours are the effects on bone marrow and thymus comparable with those on appendix. At a level of 3 mg./kg. and 5 mg./kg. similar but more marked effects are observed. HN2 produces essentially the same effects as H at comparable dose levels. Again incorporation into DNA is more significantly depressed than incorporation into nRNA or cRNA, and appendix is more readily affected than bone marrow or thymus.

TABLE I.—*Effect of di-(2-chloroethyl) Sulphide (H) on the Incorporation of $^{32}PO_4^{\equiv}$ into the Nucleic Acids of Rabbit Tissues* In Vivo.

All results are expressed as percentages of the mean values of the controls.

Dose (mg./kg.)	Time interval between administration of H and isotope administration (hr.)	Specific activity relative to blood inorganic phosphate								
		Appendix			Bone marrow			Thymus		
		DNA	nRNA	cRNA	DNA	nRNA	cRNA	DNA	nRNA	cRNA
1·5	2	79	201	151	114	149	164	100	196	128
1·5	2	33	79	61	77	89	71	51	90	76
1·5	2	48	59	96	105	129	132	85	53	95
1·5	24	10	47	37	9	32	45	5	29	31
1·5	48	77	109	102	109	146	100	177	200	165
1·5	48	49	67	63	63	103	128	19	75	56
3·0	2	32	113	72	72	161	102	51	114	73
3·0	2	21	58	52	67	63	72	33	86	40
3·0	4	15	42	31	83	119	71	41	61	38
3·0	8	5	18	20	31	67	71	17	35	21
3·0	24	9	66	38	7	24	65	3	21	28
5·0	2	21	50	62	44	63	73	36	91	53
5·0	2	8	37	41	29	48	44	13	52	52
5·0	2	7	86	47	77	112	128	50	58	38
5·0	8	2	29	25	18	46	82	27	35	31
Control	—	100	100	100	100	100	100	100	100	100
Coefficient of variation of controls	—	26	23	25	23	29	40	35	23	40

TABLE II.—*Effect of Methyl-di-(2-chloroethyl)amine (HN2) on the Incorporation of $^{32}PO_4^{\equiv}$ into the Nucleic Acids of Rabbit Tissues* In Vivo.

All results are expressed as percentages of the mean values of the controls.

Dose (mg./kg.)	Time interval between administration of HN2 and isotope injection (hr.)	Specific activity relative to blood inorganic phosphate								
		Appendix			Bone marrow			Thymus		
		DNA	nRNA	cRNA	DNA	nRNA	cRNA	DNA	nRNA	cRNA
1·5	24	42	120	101	113	108	176	37	59	52
1·5	24	15	38	41	13	48	44	59	37	37
1·5	48	59	102	109	193	218	317	121	107	136
1·5	98	132	264	196	171	173	215	154	165	145
2·5	2	96	117	152	128	178	175	110	87	267
2·5	2	40	90	84	76	107	122	57	131	80
2·5	8	4	39	40	29	86	101	40	85	54
3·0	8	—	31	31	30	65	76	23	52	33
3·0	24	4	28	26	8	21	26	9	25	23
5·0	2	33	70	87	75	110	131	53	119	95
5·0	2	35	90	107	59	90	112	52	109	111
5·0	8	2	28	29	20	56	76	20	59	34
10·0	2	22	94	89	59	90	—	41	81	91
10·0	2	19	—	82	66	110	104	52	77	90
Control	—	100	100	100	100	100	100	100	100	100
Coefficient of variation of Control	—	26	23	25	23	29	40	35	23	40

These experiments would therefore appear to indicate that both H and HN2 exert a more marked effect on DNA synthesis than on RNA synthesis, and that this effect is demonstrable 2 to 48 hours after administration. It was not, however, possible, in view of the small number of animals which could be used for each experiment, to determine statistically whether under any conditions of dosage or time interval, H or HN2 either (*a*) completely inhibits synthesis of DNA but not of RNA, or (*b*) reduces synthesis of DNA without affecting synthesis of RNA. For this reason several experiments *in vitro* were undertaken using the Ehrlich ascites carcinoma. This tumour was employed because of its rapid rate of growth and nucleic acid synthesis, and because it is readily obtainable in large quantities. It has the additional advantage that since it consists of individual cells suspended in plasma it is particularly suitable for studies *in vitro*. Since the rabbit experiments showed no difference in effect between H and HN2 only the latter compound was used in ascites tumour experiments.

Table III shows the effect of HN2 on $^{32}PO_4^{\equiv}$ incorporation into the nucleic acids of the Ehrlich tumour during 2 hours *in vitro*. At a concentration of 50 μg./ml. incorporation into DNA, nRNA and cRNA is reduced to about 30–40 per cent of the normal level: at 20 μg./ml. similar but less dramatic effects are found. Concentrations below this level produce smaller and more variable effects. In all cases incorporation into DNA, nRNA and cRNA is depressed to about the same extent; there is no indication of a specific effect on DNA. Confirmation of these results may be seen in Table IV, which shows that the effect of HN2 on the incorporation of (^{14}C) formate into the nucleic acid bases of the tumour *in vitro* is to depress incorporation into the thymine and purines of DNA and the purines of nRNA and cRNA to an appreciable extent. (The figures obtained for the activities of the purines of DNA and cRNA shown on Table IV are so low that they must be regarded as very approximate estimates.) The observation that the isotope is incorporated under *in vitro* conditions much more extensively into DNA thymine than into the nucleic acid purines has been discussed elsewhere (Smellie, Thomson, Goutier and Davidson, 1956).

TABLE III.—*Effect of Methyl-di-(2-chloroethyl)amine (HN2) on Incorporation of $^{32}PO_4^{\equiv}$ into the Nucleic Acids of the Ehrlich Ascites Carcinoma In Vitro*

(Incorporation time: 2 hr. Isotope concentration: 10 μc./ml.)

HN2 concentration (μg./ml.)	Specific activity (counts/min./100 μg. P.)		
	DNA	nRNA	cRNA
0	10,030	21,432	1,676
20	6,025	15,366	1,325
50	3,600	7,462	732

These results obtained *in vitro* differ from the results of the rabbit experiments *in vivo* in two respects: the effects are produced only at relatively high concentrations of HN2 (20 to 50 μg./ml. compared with 1·5 to 5 mg./kg.) and no indication is to be found of a specific effect on DNA. For these reasons several experiments were carried out using the MC1M ascites sarcoma in order to determine whether this tumour behaves differently from the Ehrlich carcinoma. As the results

TABLE IV.—*Effect of Methyl-di-(2-chloroethyl)amine (HN2) on Incorporation of (^{14}C)formate into the Nucleic Acids of the Ehrlich Ascites Carcinoma* In Vitro

(Incorporation time : 2 hr. Isotope concentration : 3 μc./ml.)

HN2 concentration μg./ml.	Specific activity (counts/min./μmole base)						
	DNA			nRNA		cRNA	
	Adenine	Guanine	Thymine	Adenine	Guanine	Adenine	Guanine
0	53	83	3140	1236	2737	124	628
10	40	56	2221	974	1175	80	150
20	69	92	1455	655	920	35	12
50	25	21	896	340	566	0	0

in Tables V and VI show, the effects of HN2 on incorporation of $^{32}PO_4^{\equiv}$ (Table V and (^{14}C(formate (Table VI) into the nucleic acids of the MC1M tumour are similar to those found in the case of the Ehrlich tumour. As before, the effect is evident only at high concentrations of HN2 (20 to 50 μg./ml.) and incorporation into DNA, nRNA and cRNA is diminished to approximately the same extent.

TABLE V.—*Effect of Methyl-di-(2-chloroethyl)amine (HN2) and a Nucleoside Mixture* on Incorporation of $^{32}PO_4^{\equiv}$ into the Nucleic Acids of the MC1M Mouse Ascites Sarcoma*

(Incorporation time : 2 hr. Isotope concentration : 10 μc./ml.)

HN2 concentration μg./ml.	Additions	Specific activity (counts/min./100 μg. P)		
		DNA	nRNA	cRNA
0	Nil	1,860	39,252	6,244
50	,,	617	20,130	3,641
0	Nucleosides	1,865	42,986	8,659
50	,,	811	19,131	4,653

* The nucleoside mixture provided 0·5 μmole adenosine, guanosine, uridine, cytidine and thymidine per ml. of cell suspension.

TABLE VI.—*Effect of Methyl-di-(2-chloroethyl)amine (HN2) and Mouse Liver Extract on Incorporation of (^{14}C)formate into the Nucleic Acids and Acid-soluble Adenine Compounds of the MC1M Mouse Ascites Sarcoma* In Vitro

(Incorporation time : 2 hr. Isotope concentration : 3 μc./ml.)

HN2 concentration μg./ml.	Additions	Specific activity (counts/min./μmole base)							
		DNA			nRNA		cRNA		Acid-soluble adenine compounds
		Adenine	Guanine	Thymine	Adenine	Guanine	Adenine	Guanine	
0	Nil	0	0	322	177	288	—	—	2,510
50	,,	0	0	123	100	143	0	0	3,435
0	Mouse liver extract	44	80	491	1,226	812	131	0	13,085
50	Ditto	0	0	316	875	635	129	0	17,100

In the light of the work of Grunberg-Manago, Ortiz and Ochoa (1956) and of Kornberg (1957), it seems probably that nucleic acid biosynthesis may proceed in the following stages :

(*a*) synthesis of purine and pyrimidine nucleotides,
(*b*) their phosphorylation to nucleoside di- or triphosphates,
(*c*) condensation of such nucleotides to form polynucleotides.

To determine which of these stages might be affected by HN2, an experiment was performed to test whether the effect of HN2 on incorporation of $^{32}PO_4^{\equiv}$ into the nucleic acids of the Ehrlich tumour could be reversed by addition of a mixture of adenosine, guanosine, uridine, cytidine, and thymidine. The results in Table V indicate that the nucleoside mixture only slightly reduces the effect of HN2. Presumably, therefore, HN2 must act chiefly on the further phosphorylation of nucleosides or on the mechanism of the combination of mononucleotides to form nucleic acids. This conclusion is supported by the results of an experiment to determine whether mouse liver extract, which is known to stimulate incorporation of formate into nucleic acid purines in the Ehrlich ascites carcinoma (Smellie *et al.*, 1956), diminishes the effect of HN2. Table VI shows that both in presence and in absence of liver extract HN2 reduces incorporation of (^{14}C)formate into DNA thymine by almost 50 per cent. It produces a similar reduction in incorporation into nRNA purines although incorporation into acid-soluble purines is increased It is clear, therefore, that HN2 does not depress the biosynthesis of the purine nucleotides. Incorporation into cRNA and DNA purines in systems of this type is too small to be accurately measured. Finally, the fact that HN2 depresses incorporation of (8-^{14}C)adenine into DNA, nRNA and cRNA to a similar extent (Table VII) is also consistent with its effect being on the utilization of nucleotide units rather than on their synthesis.

TABLE VII.—*Effect of Methyl-di-(2-chloroethyl)amine (HN2) on Incorporation of (8-^{14}C)adenine into the Nucleic Acid Adenine of the MC1M Ascites Sarcoma* In Vitro

(Incorporation time : 2 hr. Isotope concentration : 3 μc./ml.)

HN2 concentration μg./ml.	Specific activity (counts/min./μmole base) DNA Adenine	nRNA Adenine	cRNA Adenine	Acid-soluble adenine compounds
0	79	3,150	309	42,300
20	34	2,042	209	52,915
50	29	1,275	156	45,145

Since neither the Ehrlich or the MC1M tumours appeared to be so sensitive to HN2 *in vitro* as the rabbit tissues had proved to be *in vivo*, it seemed important to determine whether their sensitivity could be increased. For this purpose Ehrlich ascites carcinoma cells were separated from their plasma by centrifuging, and after being washed several times in Krebs-Ringer bicarbonate buffer they were resuspended in the same medium. Such washed cell suspensions are still capable of rapidly incorporating labelled precursors into their nucleic acids. The effect

of HN2 on incorporation of $^{32}PO_4^{\equiv}$ into such preparations is shown in Table VIII. The observation that even a concentration of 5 μg./ml. produces a marked reduction, suggests that the washed cells are much more sensitive than are unreated cells. Nevertheless, both DNA and RNA are affected to about the same extent.

TABLE VIII.—*Effect of Methyl-di-(2-chloroethyl)amine (HN2) on Incorporation of $^{32}PO_4^{\equiv}$ into the Nucleic Acids of Washed Ehrlich Ascites Carcinoma Cells Incubated in Krebs-Ringer Bicarbonate Solution*

(Incorporation time : 2 hr. Isotope concentration : 8 μc./ml.)

HN2 concentration μg./ml.	Specific activity (counts/min./100 μg. P) DNA	RNA
0	17,122	79,650
5	9,811	47,424
20	8,757	21,198
50	4,230	7,702

The effect of HN2 on (^{14}C)formate incorporation in washed cells is shown in Table IX. At a concentration of 10 μg./ml. a marked depression of incorporation into DNA thymine is produced. Incorporation into DNA and RNA purines, on the other hand, is virtually unaffected ; but this apparent lack of inhibition is presumably related to the fact that the HN2 markedly increases incorporation of formate into acid-soluble purines, so that, in fact, the incorporation of acid-soluble purines into nucleic acids is diminished to the same extent as incorporation of (^{14}C)formate into DNA thymine. This point is clearly illustrated in Table X which shows the effect of HN2 and 5-amino-4-imidazolecarboxamide (AICA) riboside on incorporation of (^{14}C)formate into washed cells. AICA riboside has already been shown (Thomson, Smellie and Harrington, 1957) to bring about a sharp increase in incorporation of (^{14}C)formate into acid-soluble and nucleic acid purines of the Ehrlich tumour without affecting incorporation into DNA thymine.

TABLE IX.—*Effect of Methyl-di-(2-chloroethyl)amine (HN2) on Incorporation of (^{14}C)formate into the Nucleic Acid Bases and Acid-soluble Adenine of Washed Ehrlich Ascites Carcinoma Cells Incubated in Krebs-Ringer Bicarbonate Solution*

(Incorporation time : 2 hr. Isotope concentration : 3 μc./ml.)

HN2 concentration μg./ml.	Specific activity (counts/min./μmole base) DNA Adenine	DNA Thymine	RNA Adenine	Acid-soluble adenine compounds
0	7	4961	107	1493
2	7	4969	112	1817
5	6	4353	113	2607
10	7	1461	102	5132

The results in Table X show that AICA riboside has a similar effect on washed cells. In its absence the effect of HN2 is to depress incorporation of (^{14}C)formate into DNA thymine to about 5 per cent of its original level. Incorporation into

RNA purines is diminished to 50 per cent of its original level, but simultaneously incorporation into the acid-soluble adenine is *increased* four-fold so that incorporation of acid-soluble purines into nucleic acids is diminished to about 10 per cent of its original level, i.e. incorporation of formate into DNA thymine and of acid-soluble adenine into RNA are diminished to about the same extent. In presence of AICA riboside HN2 diminishes, instead of stimulating, incorporation of (^{14}C) formate into acid-soluble adenine and, in this case, incorporation of formate into DNA thymine and into DNA and RNA purines is diminished uniformly to 5 to 15 per cent of its normal value.

TABLE X.—*Effect of Methyl-di-(2-chloroethyl)amine (HN2) and 5-amino-4-imidazolecarboxamide Riboside (AICA Riboside) on Incorporation of (^{14}C)formate into the Nucleic Acids and Acid-soluble Bases of Washed Ehrlich Ascites Carcinoma Cells Incubated in Krebs-Ringer Bicarbonate Solution*

(Incorporation time : 2 hr. Isotope concentration : 3 μc./ml.)

HN2 concentration μg./ml.	Addition	Specific activity (counts/min./μmole base) DNA Adenine	DNA Guanine	DNA Thymine	RNA Adenine	RNA Guanine	Acid-soluble adenine compounds
0	Nil	36	193	6,300	315	645	4,295
20	„	3	—	350	182	318	16,167
0	AICA riboside	1,762	2,880	6,746	13,005	9,790	184,350
20	„ „	72	182	309	1,928	1,296	119,675

None of these experiments *in vitro* gives any clear indication that HN2 has a specific effect on DNA. Tables XI and XII show the results of experiments to determine whether such an effect could be demonstrated in washed cells if the incubation time were varied. When (^{14}C)formate was used as a precursor it was found that HN2 caused a complete cessation of incorporation into both RNA and DNA after the first hour (Table XI) although incorporation with the acid-soluble purines continued after this time interval. Incorporation of (8-^{14}C)adenine

TABLE XI.—*Effect, at Various Time Intervals, of Methyl-di-(2-chloroethyl)-amine (HN2) on Incorporation of (^{14}C)formate into the Nucleic Acids and Acid-soluble Adenine Compounds of Washed Ehrlich Ascites Carcinoma Cells Incubated in Krebs-Ringer Bicarbonate Buffer*

(Isotope concentration : 4 μc./ml.)

HN2 concentration μg./ml.	Time (hr.)	Specific activity (counts/min./μmole base) DNA Adenine	DNA Thymine	RNA Adenine	Acid-soluble adenine compounds
0	1	8	2325	76	1190
0	2	16	3996	137	1628
0	4	41	5339	287	—
20	1	3	409	40	3079
20	2	7	447	63	5530
20	4	4	429	66	—

(Table XII) into nucleic acids, on the other hand, was not completely abolished by HN2 although it was greatly diminished. Incorporation of both precursors into the acid-soluble nucleotide adenine was increased in presence of HN2.

TABLE XII.—*Effect, at Various Time Intervals, of Methyl-di-(2-chloroethyl)-amine (HN2) on Incorporation of (8-^{14}C)adenine into the Nucleic Acids and Acid-soluble Adenine Nucleotides of Washed Ehrlich Ascites Carcinoma Cells Incubated in Krebs-Ringer Bicarbonate Buffer*

(Isotope concentration : 3 μc./ml.)

HN2 concentration μg./ml.	Time (hr.)	Specific activity (counts/min./μmole base) DNA Adenine	RNA Adenine	Acid-soluble nucleotide adenine*
0	1	56	710	14,807
0	2	116	1,620	31,245
0	4	265	2,800	54,270
20	1	26	358	28,985
20	2	28	724	75,660
20	4	48	1,375	140,425

* Prior to hydrolysis and chromatography, the acid-soluble fraction was extracted 8 times with *n*-butanol to remove free adenine and adenosine.

Since none of these experiments on ascites tumours gave any indication that HN2 has a specific effect on incorporation of labelled precursors into DNA *in vitro*, an attempt was made to determine whether this was due either to the nature of the ascites tumour or to the limitations inherent in experiments on surviving tissues. For this purpose the effects of HN2 on the DNA and RNA content of growing cultures of Earle's " L " cells were investigated.

A preliminary experiment to determine a suitable mustard concentration showed that the " L " cells are much more sensitive to HN2 than ascites cells. At a concentration of HN2 as low as 1 μg./ml. considerable cell death occurred as shown by the fact that the DNA content of the cultures was reduced to about 65 per cent of the initial value. At concentrations of HN2 of from 0·25 to 0·5 μg./ml. growth continued but the rate of synthesis of nucleic acids was reduced (Table XIII), DNA being affected to a greater extent than RNA so that the ratio of RNA : DNA per culture increased as the mustard concentration was increased.

TABLE XIII.—*Effect of Methyl-di-(2-chloroethyl)amine (HN2) on the Nucleic Acids of Growing Cultures of Earle's " L " Cells*

Figures are given in terms of amount per flask.

HN2 concentration μg./ml.	DNA μg.	RNA μg.	Ratio RNA/DNA
Zero time control	50·8	64·8	1·28
0	128.8	137·5	1·07
0·25	81·2	103·3	1·27
0·33	70·2	97·5	1·39
0·5	57·8	91·3	1·58

The figures given, except the initial control values, are the mean value for 4 replicate cultures.

DISCUSSION

It is widely believed that mustards have a specific effect on the metabolism of DNA as distinct from RNA, but there is little published evidence to support this point of view. The experiments reported here were carried out to ascertain whether such a specific effect could be demonstrated in mammalian tissues.

No evidence for a specific effect of mustards on DNA synthesis was obtained in experiments with the Ehrlich mouse ascites carcinoma (Tables III–IV, Tables VIII–X) or the MC1M mouse ascites sarcoma (Tables V–VII). Other workers using similar materials have also failed to detect a differential effect of nitrogen mustard on isotope incorporation into DNA and RNA. Adenocarcinoma 755, when treated *in vivo* with HN2, does not show any specific effect of the mustard on the incorporation of $^{32}PO_4^{\equiv}$ into DNA (Davidson and Freeman, 1955). Results of a similar nature have been reported by LePage and Greenlees (1955) for the incorporation of (2-^{14}C)glycine into the nucleic acids of ascites cells. Heidelberger and Keller (1955) failed to detect a specific effect of HN2 on the uptake of (^{14}C)-formate into the DNA of Flexner-Jobling carcinoma of the rat.

On the other hand, in the present series of experiments some evidence for a specific effect of H and HN2 has been obtained in the intact rabbit and in cells grown in tissue culture. The results obtained from the rabbit experiments (Tables I–II) suggest that, while the mustards depress incorporation of $^{32}PO_4^{\equiv}$ into both DNA and RNA, depression of uptake into DNA is more marked than that into RNA. These results are in agreement with those obtained by Lowrance and Carter (1950) in a similar but less extensive series of experiments with rabbits.

In the tissue culture experiments on the total amount of DNA and RNA present in cultures of control cells and cells treated with concentrations of nitrogen mustard in the range 0·25 to 0·5 μg./ml. there is a progressive increase in the ratio of RNA : DNA with increasing mustard concentration. This change in the RNA : DNA ratio may be interpreted as evidence that HN2 curtails DNA synthesis to a greater extent than RNA synthesis.

If the biosynthesis of the nucleic acids is considered as being a three-stage process involving synthesis of mononucleotides, their phosphorylation to give nucleoside di- or tri-phosphates, and condensation of the di- or tri-phosphates to form polynucleotides, the evidence so far obtained suggests that HN2 inhibits the process at either the second or third stage. Support for this suggestion may be derived from studies on the effect of mustards on enzymes, *in vitro*. It has been shown that the majority of enzymes are relatively unaffected by the mustards but there is a smaller group of sensitive enzymes consisting mainly of phosphokinases including those necessary for the phosphorylation or dephosphorylation of adenylic residues (Needham, 1948).

With MC1M cells 50 μg./ml. HN2 depresses $^{32}PO_4^{\equiv}$ incorporation into DNA, nRNA and cRNA (Table V) and also decreases formate incorporation into DNA thymine without producing any clear-cut effect on formate incorporation into nucleic acid purines (Table VI). This means that the inhibition of $^{32}PO_4^{\equiv}$ incorporation is not due to inhibition of purine synthesis. Further evidence for this point of view is found in the experiments showing that neither mouse liver extract (Table VI), AICA riboside (Table X), nor a mixture of nucleosides (Table V) completely reverses the effect of HN2 on incorporation.

In experiments in which the incorporation of labelled formate or adenine into the acid-soluble adenine has been measured, the results show that the specific activities of adenine from the acid-soluble fractions of mustard-treated cells were not lower, and usually much higher, than those of adenine from the acid-soluble fractions of control cells (Tables XI and XII). Thus under conditions in which there is clear-cut evidence of the inhibition of nucleic acid biosynthesis, the synthesis of acid-soluble purine nucleotides is not diminished but enhanced.

These views on the mechanism of action of nitrogen mustard are in agreement with those put forward by Goldthwait (1952) who suggested on the basis of his study of the effect of HN2 on the simultaneous incorporation of (^{14}C) formate and (1 : 3–$^{15}N_2$)adenine into the small intestine of the rat, that HN2 did not inhibit the formation of the purine skeleton.

The general conclusion to be drawn from the present studies is that although H and HN2 may exercise a more pronounced inhibitory action on the metabolism of DNA than of RNA, especially in the intact animal, there is no evidence to show that mustard at any concentration exercises a specific effect on DNA metabolism only.

We should like to thank colleagues at the Ministry of Supply Chemical Defence Experimental Establishment at Porton for helpful discussions, Dr. John Paul of the H.E.R.T. Tissue Culture Laboratory of this Department for valuable help and guidance in the experiments with " L " cells, Dr. G. J. Popjak of Hammersmith Hospital for a gift of mice bearing the Ehrlich ascites carcinoma, Dr. G. Klein of Stockholm for a gift of mice bearing the MC1M sarcoma, and Messrs. Boots Pure Drug Company of Nottingham for a gift of nitrogen mustard.

SUMMARY

In an attempt to determine whether sulphur and nitrogen mustards exerted a specific effect on DNA metabolism, the effect of H and HN2 on the nucleic acid metabolism of a number of mammalian systems was investigated.

1. In experiments with rabbits *in vivo* mustard exercised a more pronounced inhibitory effect on DNA metabolism than on RNA metabolism. Further evidence for a selective effect on DNA was obtained from experiments with Earle's " L " strain cells grown in tissue culture.

2. No evidence for a differential effect of HN2 on DNA metabolism was obtained from experiments with Ehrlich mouse ascites carcinoma cells incubated, *in vitro*, in ascitic plasma or from MC1M mouse ascites sarcoma cells incubated *in vitro* in ascitic plasma.

3. The evidence obtained suggests that nitrogen mustard depresses nucleic acid synthesis by inhibiting either the phosphorylation of mononucleotides to form di- or tri-phosphates or the condensation of these di- or tri-phosphates to give polynucleotides rather than by inhibiting the biosynthesis of mononucleotides.

REFERENCES

Bodenstein, D. and Kondritzer, A. A.—(1948) *J. exp. Zool.*, **107**, 109.

Cohen, P. P.—(1951) 'Manometric Techniques and Tissue Metabolism', edited by W. W. Umbreit, R. H. Burris and J. J. Stauffer. 2nd ed. Minneapolis (Burgess Publishing Co.), p. 193.

Davidson, J. D. and Freeman, B. F.—(1955) *Cancer Res., Suppl.*, **3**, 97.

GOLDTHWAIT, D. A.—(1952) *Proc. Soc. exp. Biol. N.Y.*, **80**, 503.
GRUNBERG-MANAGO, M., ORITZ, P. J. AND OCHOA, S.—(1956) *Biochim. biophys. Acta*, **20**, 269.
HANKS, J. H. AND WALLACE, R. E.—(1949) *Proc. Soc. exp. Biol. N.Y.*, **71**, 196.
HEIDELBERGER, C. AND KELLER, R. A.—(1955) *Cancer Res. Suppl.*, **3**, 106.
HERRIOTT, R. M.—(1950–51) *J. gen. Physiol.*, **34**, 761.
KLEIN, G.—(1951) *Exp. Cell Res.*, **2**, 518.
KORNBERG, A.—(1957) 'The Chemical Basis of Heredity', edited by W. D. McElroy and B. Glass. Baltimore (Johns Hopkins Press), p. 579.
LEPAGE, G. A. AND GREENLEES, J. L.—(1955) *Cancer Res. Suppl.*, **3**, 102.
LOWRANCE, P. B. AND CARTER, C. E.—(1950) *J. cell comp. Physiol*, **35**, 387.
NEEDHAM, D. M.—(1948) *Biochem. Soc. Symp., No.* 2, p. 16.
PAUL, J.—(1958) *Analyst* (in Press).
PHILIPS, F. S.—(1950) *Pharmacol. Rev.*, **2**, 281.
SANFORD, K. K., EARLE, W. R. AND LIKELY, G. D.—(1948) *J. nat. Cancer Inst.*, **9**, 229.
SCHULMAN, M. P., SONNE, J. C. AND BUCHANAN, J. M.—(1952) *J. biol. Chem.*, **196**, 499.
SMELLIE, R. M. S., HUMPHREY, G. F., KAY, E. R. M. AND DAVIDSON, J. N.—(1955) *Biochem. J.*, **60**, 177.
Idem, THOMSON, R. Y. AND DAVIDSON, J. N.—(1958) *Biochem. biophys. Acta* (in Press).
Idem, THOMSON, R. Y., GOUTIER, R. AND DAVIDSON, J. N.—(1956) *Biochim. biophys. Acta*, **22**, 585.
THOMSON, R. Y., SMELLIE, R. M. S. AND HARRINGTON, H.—(1957) *Biochem. J.*, **67**, 17.

THE DISTRIBUTION OF POLYCYCLIC HYDROCARBONS WITHIN THE CELLS OF SOME MOUSE AND RAT TISSUES

G. CALCUTT

From the Department of Cancer Research, Mount Vernon Hospital and the Radium Institute, Northwood, Middlesex

Received for publication January 29, 1958

THE association of 3 : 4 benzpyrene with the major morphological components of the liver cells from animals treated with this hydrocarbon has already been described by Calcutt and Payne (1953, 1954*a*, 1954*b*). It has also been shown by Fiala, Sproul and Fiala (1955) that benzpyrene becomes bound to various components of cells from the skin of day-old mice after painting with solutions of the hydrocarbon.

So far no attempt has been made to correlate the intracellular distribution of the polycyclic hydrocarbons with their biological activity. To further such an attempt a study of the intracellular behaviour of a number of hydrocarbons has been made.

As the majority of experiments concerned with carcinogenesis have been done using rats or mice the present work has been limited to consideration of tissues taken from these two species. The actual choice of tissues for experiment has further been limited by considerations of ease of application of the hydrocarbon and the suitability of the tissue for fractionation purposes. The tissues finally chosen for experiment were: liver, kidney and the skin of day-old animals. The rodent liver is not normally regarded as being susceptible to hydrocarbon carcinogenesis, although the evidence gathered by Berman (1951) indicates that this organ can respond to polycyclic compounds by tumour formation. Similarly, the kidney does not seem to be particularly susceptible to hydrocarbons, but Ilfeld (1936) obtained tumours after the insertion of pellets of 1 : 2 : 5 : 6 dibenzanthracene into the kidneys of mice. From the data summarised by Hartwell (1951) it is apparent that adult mouse skin is highly susceptible to the carcinogenic activity of polycyclic compounds but that adult rat skin is refractory. With regard to the skin from very young animals the work of Shimkin and Mider (1941) and Rous and Smith (1945) indicates that young mouse skin is susceptible to methylcholanthrene carcinogenesis, whilst Fiala, Sproul and Fiala (1955) found tumours to occur in very young mice after painting with benzpyrene. There appear to be no records in the literature of attempts to induce skin tumours in very young rats.

It is unfortunate that adult rodent skin is not suitable material for cell fractionation experiments. Weist and Heidelberger (1953) found that after homogenisation of mouse skin only two fractions could be separated by centrifugation. One comprised unbroken cells and particulate components and the other was a clear supernatant fraction. Fiala, Sproul and Fiala (1955) showed that the skin of day-old mice could be successfully separated into cellular components and Calcutt (1957) has since found that young rat skin can be handled similarly.

It is on the basis of these findings that the skin from very young animals has been chosen for the present work.

The hydrocarbons used were anthracene and phenanthrene as non carcinogens, 1 : 2 benzanthracene as a weak carcinogen and 1 : 2 : 5 : 6 dibenzanthracene as a moderately active carcinogen. Data already exists in the case of the highly active carcinogen—3 : 4 benzpyrene.

EXPERIMENTAL

All mouse tissues used in these experiments were taken from RIII strain animals and all rat tissues from animals of the Wistar strain.

For experiments involving skin the hydrocarbons were applied as solutions in acetone, this solvent being chosen on the basis of Pullinger's (1940) statement that acetone does not affect the microscopic structure of mouse skin. In the case of liver and kidney the hydrocarbons were injected intravenously via the tail veins of the animals employed. Anthracene, 1 : 2 benzanthracene and 1 : 2 : 5 : 6 : dibenzanthracene were used as colloids in distilled water, these being prepared by dropping acetone solutions of the hydrocarbons into distilled water and then removing the acetone under reduced pressure. All attempts at preparing colloids of phenanthrene were unsuccessful and ultimately this hydrocarbon was used as a microcrystalline suspension. This was prepared as follows : dried human plasma was reconstituted with distilled water and sufficient acetone added to precipitate the proteins. These were filtered off and an acetone solution of phenanthrene was added to the filtrate. The acetone was boiled off under reduced pressure leaving a suspension of microcrystals of phenanthrene in a straw-coloured liquid. The crystals were centrifuged off, resuspended in distilled water and collected again by centrifugation. Shaking with distilled water gave a suspension of microcrystals which was satisfactory for injection purposes and was well tolerated by rats and mice.

After treatment with the hydrocarbons the animals were killed at intervals up to 24 hours, the required tissue was taken and homogenised in either 1 per cent citric acid solution or 0·88 M sucrose or Tyrode solution. After straining to remove any fibrous material or unbroken cells the homogenate was separated into nuclei, mitochondria, microsomes and supernatant by centrifugation as described by Calcutt and Payne (1954*a*). The particulate fractions were resuspended in the same dispersing medium as was used for the initial separation and centrifuged down again. After this washing procedure they were ready for examination for the presence of the hydrocarbon.

Fractions derived from liver and kidney were subjected to three different successive procedures to determine the presence of the hydrocarbon. First they were extracted with one or more non-polar solvents such as benzene or cyclohexane to remove any loosely adherent hydrocarbon. These extractions were continued until no further fluorescent material was removed. The fractions were then extracted with acetone to remove any lightly bound hydrocarbon, the extractions again being continued till no further fluorescent material was removed. Finally the fractions were hydrolysed to determine any tightly bound hydrocarbon.

The usual hydrolytic procedure for the recovery of bound hydrocarbons from tissues is to reflux with caustic potash, zinc dust and toluene. This was used on whole liver homogenates from animals which had received either anthracene or phenan-

threne intravenously. Although hydrolysis was continued for as long as three days hydrocarbon was only recovered in a number of cases and no consistent results were obtained. As an alternative tryptic digestion was tried but proved completely ineffective. Attempts at hydrolysis with concentrated sulphuric acid were unsuccessful as the resulting tarry residues finally obtained could not be satisfactorily extracted with any suitable organic solvent. Finally, a reductive hydrolytic method described by Fieser (1941) was tried. This was to reflux in hydrochloric acid in the presence of red phosphorus and potassium iodide. A number of trial runs showed the method to be both efficient and repeatable. Based on these trials the technique was standardised as being ; to reflux in 50 per cent hydrochloric acid with an obvious excess of red phosphorus and potassium iodide, these agents being used in the proportion of 6 : 2·5 by weight. Hydrolysis and release of the hydrocarbon was normally complete in 1–1½ hours. Under these conditions the entire hydrocarbon present was found to steam distil and collect in the condenser, from which it could be washed in a very pure state. Originally used with anthracene and phenanthrene this hydrolytic procedure was later successfully used with 1 : 2 benzanthracene and 1 : 2 : 5 : 6 dibenzanthracene.

In the case of skin a complication ensued from the presence of free hydrocarbon which had dried on the skin surface. This could be removed by washing with organic solvents but such a procedure caused hardening of the skin and rendered subsequent homogenisation difficult. The system was adopted of preparing the fractions as normally and then washing repeatedly with acetone till no further hydrocarbon was removed. This left fractions containing bound hydrocarbon only, and this was then released by hydrolysis as described above. All results obtained in relation to skin therefore only refer to bound hydrocarbon.

Final determinations of the hydrocarbons were by absorption spectroscopy, spectra being examined over the range from 220 mμ upwards. In the case of cyclohexane extracts direct spectroscopy on the extracts was possible. Benzene extracts were dried down and the residues taken up in ethyl alcohol or cyclohexane for spectroscopy. Acetone extracts were dried down and the residues taken up in cyclohexane. This was dried over anhydrous sodium sulphate and passed through a chromatography column of alumina. Any hydrocarbon present was held as a distinct band on the alumina and was eluted with ethyl alcohol for spectroscopy. The condensers from hydrolyses were washed out with cyclohexane and direct spectroscopy done on the solutions so obtained.

RESULTS

These are recorded individually for the different hydrocarbons used, as below:—

Anthracene.—Mice received 0·5 mg. of hydrocarbon in 0·5 c.c. of distilled water intravenously. Rats received 1·0 mg. of hydrocarbon in 1·0 c.c. of distilled water intravenously. No free hydrocarbon could be extracted from whole homogenates or separated cell fractions of either mouse or rat liver or kidneys by washing with non-polar solvents. Extractions of liver or kidney homogenates or fractions with acetone, ethyl ether or ethyl alcohol also yielded no hydrocarbon. Results obtained with hydrolysed fractions are shown in Table I for liver fractions and in Table II for kidney fractions. In all cases the amount of hydrocarbon recovered was small, but adequate for spectroscopic identification.

For experiments with skin animals were painted twice at intervals of a few minutes with a saturated solution (approximately 0·5 per cent) of anthracene in acetone. The findings in respect of rat and mouse skin are shown in Table III.

Phenanthrene.—Mice received approximately 0·5 mg. of micro-crystals in 0·5 c.c. of distilled water intravenously and rats 1·0 mg. of micro-crystals in 1·0 c.c. of distilled water intravenously. No hydrocarbon was recoverable from whole homogenates or separated fractions of mouse or rat liver or kidneys by extraction with either non-polar or polar solvents. Results in respect of hydrolysed fractions are given in Table IV for liver and Table V for kidney.

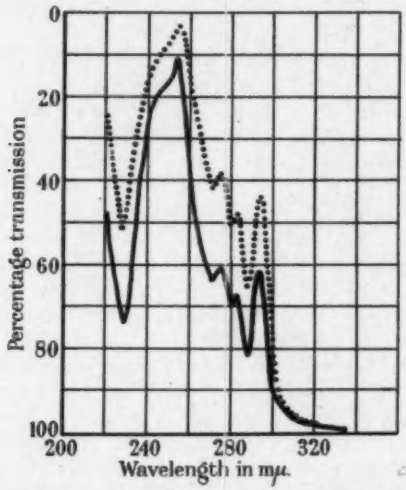


FIG. 1.—Absorption spectrum of phenanthrene obtained from rat skin microsomes extracted in citric acid 21 hours after painting with the hydrocarbon.

Full line—control spectrum of phenanthrene in cyclohexane.
Dotted line—spectrum of recovered phenanthrene in cyclohexane.

The animals used for skin experiments were painted with a 1 per cent solution of phenanthrene in acetone. The results of these experiments are shown in Table VI.

Results obtained in the above experiments exactly parallel those obtained with anthracene. Recoveries of phenanthrene were, however, rather better than in similar experiments with anthracene. A typical example of the absorption spectrum of recovered hydrocarbon is given in Fig. 1. This is of hydrocarbon as washed out of the condenser after hydrolysis and attests to the efficiency of the method in respect of the return of pure material.

1 : 2 *Benzanthracene.*—Mice received 0·5 mg. of hydrocarbon in 0·5 c.c. of distilled water intravenously and rats 1·0 mg. in 1·0 c.c. of distilled water intravenously. No hydrocarbon was recoverable by the extraction of whole homogenates or separated cell fractions of rat or mouse liver or kidneys with cyclohexane or benzene. At intervals up to 12 hours after injection small amounts of 1 : 2

benzanthracene were recoverable from nuclei, mitochondria, microsomes and supernatant by extraction with acetone. After 12 hours the hydrocarbon was still extractable from the particulate fractions but not from the supernatant. Results obtained from hydrolyses are given in Table VII for liver and Table VIII for kidney.

For skin experiments the animals were painted with a 1 per cent solution of 1 : 2 benzanthracene in acetone. Results obtained are given in Table IX.

Recoveries of 1 : 2 benzanthracene were poor, often being barely sufficient for spectroscopic identification. In Fig. 2 is illustrated the spectrum obtained by utilising the entire hydrocarbon recovered from the mitochondria of the skins of 40 mice 4 hours after painting with 1 : 2 benzanthracene. In this particular case some background absorption due to contaminant is also present.

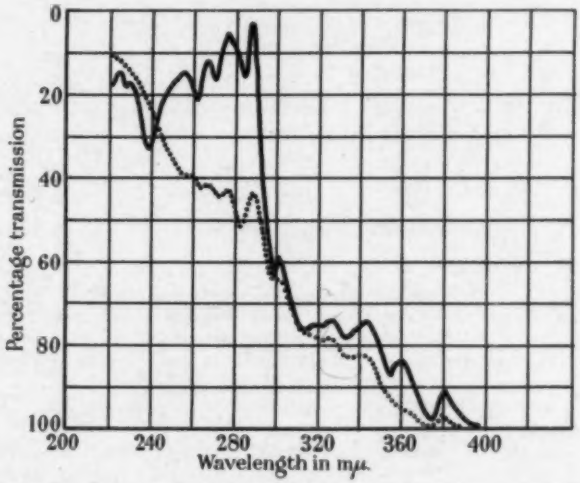


FIG. 2.—Absorption spectrum of 1 : 2 benzanthracene recovered from mouse skin mitochrondria 4 hours after application of the hydrocarbon.

Full line—control spectrum of 1 : 2 benzanthracene in cyclohexane.
Dotted line—spectrum of recovered 1 : 2 benzanthracene in cyclohexane.

This compound differs from the two previously considered cases in that all four cell fractions contain the hydrocarbon and part of this material is extractable with a polar solvent.

1 : 2 : 5 : 6 *Dibenzanthracene.*—For liver and kidney experiments mice received an intravenous injection of 0·5 mg. in 0·5 c.c. of distilled water and rats 1·0 mg. in 1·0 c.c. of distilled water. The hydrocarbon was readily extractable from whole homogenates or cell fractions with cyclohexane or benzene. After removal of this " loose " hydrocarbon further dibenzanthracene was extractable from all four cell fractions of both liver and kidney with acetone. Further hydrocarbon was then obtained by hydrolysis, the findings being shown in Table X for liver and Table XI for kidney. Recoveries were excellent and far superior in quantity

TABLE I.—*Distribution of Anthracene in Liver Cell Fractions from Mice and Rats*

	Number of animals	Sex	Time after injection (hours)	Extraction medium	Nuclei	Mitochrondria	Microsomes	Supernatant
Mice	11	F.	2	1% citric acid	—	—	An	An
	9	M.	4	Ditto	—	—	An	An
	10	F.	16	Tyrode	—	—	An	An
Rats	1	F.	1½	1% citric	—	—	An	An
	2	M.	18	Ditto	—	—	An	An

—, hydrocarbon absent. An, anthracene present.

TABLE II.—*Distribution of Anthracene in Kidney Cell Fractions from Mice and Rats*

	Number of animals	Sex	Time after injection (hours)	Extraction medium	Nuclei	Mitochondria	Microsomes	Supernatant
Mice	11	F.	2	1% citric acid	—	—	An	An
	9	M.	4	Ditto	—	—	An	An
	10	F.	16	Tyrode	—	—	An	An
Rats	1	F.	1½	1% citric acid	—	—	An	An
	2	M.	18	Ditto	—	—	An	An

—, hydrocarbon absent. An, anthracene present.

TABLE III.—*Distribution of Anthracene in Cell Fractions from Skin of Day-old Mice and Rats*

	Number of animals	Time after painting (hours)	Nuclei	Mitochondria	Microsomes	Supernatant
Mice	38	3	—	—	An	An
	14	16	—	—	An	? An
Rats	9	3	—	An	An	? An
	12	24	—	An	—	—

—, hydrocarbon absent. An, anthracene present. ? An, amount too small for positive identification. In all cases the cell fractions were isolated in 1 per cent citric acid.

TABLE IV.—*Distribution of Phenanthrene in Liver Cell Fractions from Mice and Rats*

	Number of animals	Sex	Time after injection (hours)	Nuclei	Mitochondria	Microsomes	Supernatant
Mice	10	F.	3	—	—	Ph	Ph
	8	M.	16	—	—	Ph	Ph
Rats	1	M.	2	—	—	Ph	Ph
	1	F.	17¼	—	—	Ph	Ph

—, hydrocarbon absent. Ph, phenanthrene present. All fractions were isolated in 1 per cent citric acid.

TABLE V.—*Distribution of Phenanthrene in Kidney Cell Fractions from Mice and Rats*

	Number of animals	Sex	Time after injection (hours)	Nuclei	Mitochondria	Microsomes	Supernatant
Mice	10	F.	3	—	—	Ph	Ph
	8	M.	16	—	—	Ph	Ph
Rats	1	M.	2	—	—	Ph	Ph
	1	F.	17½	—	—	Ph	Ph

—, hydrocarbon absent. Ph, phenanthrene present. All fractions were isolated in 1 per cent citric acid.

TABLE VI.—*Distribution of Phenanthrene in Cell Fractions from the Skin of Day-old Mice and Rats*

	Number of animals	Time after painting (hours)	Nuclei	Mitochondria	Microsomes	Supernatant
Mice	32	4	—	—	Ph	Ph
	27	18	—	—	Ph	Ph
Rats	13	3	—	Ph	Ph	Ph
	13	21	—	Ph	Rh	—

—, hydrocarbon absent Ph, phenanthrene present. All fractions were isolated in 1 per cent citric acid.

TABLE VII.—*The Distribution of* 1 : 2 *Benzanthracene in Liver Cell Fractions from Mice and Rats*

	Number of animals	Sex	Time after injection (hours)	Extraction medium	Nuclei	Mitochondria	Microsomes	Supernatant
Mice	8	M.	2	Tyrode	Ba	Ba	Ba	Ba
	8	F.	3½	0·88 M sucrose	Ba	Ba	Ba	Ba
	10	F.	22	1% citric acid	Ba	Ba	Ba	—
Rats	1	M.	4	Ditto	Ba	Ba	Ba	Ba
	1	F.	15	Tyrode	Ba	Ba	Ba	—
	1	M.	18	„	Ba	Ba	Ba	—

—, hydrocarbon absent. Ba, 1 : 2 benzanthracene present.

TABLE VIII.—*The Distribution of* 1 : 2 *Benzanthracene in Kidney Cell Fractions from Mice and Rats*

	Number of animals	Sex	Time after injection (hours)	Extraction medium	Nuclei	Mitochondria	Microsomes	Supernatant
Mice	8	M.	2	Tyrode	Ba	Ba	Ba	Ba
	8	F.	3½	0·88 M sucrose	Ba	Ba	Ba	Ba
	10	F.	22	1% citric aicd	—	—	—	—
Rats	1	M.	4	Ditto	Ba	Ba	Ba	Ba
	1	F.	15	Tyrode	Ba	Ba	Ba	—
	1	M.	18	„	—	—	—	—

—, hydrocarbon absent. Ba, 1 : 2 benzanthracene present.

TABLE IX.—*The Distribution of* 1 : 2 *Benzanthracene within Cell Fractions from the Skin of Day-old Mice and Rats*

	Number of animals	Time after painting (hours)	Nuclei	Mitochondria	Microsomes	Supernatant
Mice	38	2	Ba	Ba	Ba	Ba
	40	4	Ba	Ba	Ba	Ba
	23	22	Ba	Ba	? Ba	? Ba
Rats	9	2	Ba	Ba	Ba	Ba
	12	6	Ba	Ba	Ba	Ba
	11	18	Ba	Ba	? Ba	Ba

Ba, 1 : 2 benzanthracene present. ? Ba, amount too small for positive identification. All fractions were isolated in 1 per cent citric acid.

TABLE X.—*The Distribution of* 1 : 2 : 5 : 6 *Dibenzanthracene in Liver Cell Fractions from Mice and Rats*

	Number of animals	Sex	Time after injection (hours)	Extraction medium	Nuclei	Mitochondria	Microsomes	Supernatant
Mice	9	M.	1½	1% citric acid	Dba	Dba	Dna	Dba
	8	F.	4	Tyrode	Dba	Dba	Dba	Dba
	8	F.	18	0·88 M sucrose	Dba	Dba	Dba	Dba
Rats	2	M.	3	1% citric acid	Dba	Dba	Dba	Dba
	2	F.	17	Tyrode	Dba	Dba	Dba	Dba

Dba, 1 : 2 : 5 : 6 dibenzanthracene present

TABLE XI.—*The Distribution of* 1 : 2 : 5 : 6 *Dibenzanthracene within Kidney Cell Fractions from Mice and Rats*

	Number of animals	Sex	Time after injection (ours)	Extraction medium	Nuclei	Mitochondria	Microsomes	Supernatant
Mice	9	M.	1½	1% citric acid	Dba	Dba	Dba	Dba
	8	F.	4	Tyrode	Dba	Dba	Dba	Dba
	8	F.	18	0·88 M sucrose	Dba	Dba	Dba	—
Rats	2	M.	3	1% citric acid	Dba	Dba	Dba	? Dba
	2	F.	17	Tyrode	Dba	Dba	Dba	—

—, hydrocarbon absent. Dba, 1 : 2 : 5 : 6 dibenzanthracene present. ? Dba, amount too small for positive identification.

TABLE XII.—*The Distribution of* 1 : 2 : 5 : 6 *Dibenzanthracene within Cell Fractions from the Skin of Day-old Mice and Rats*

	Number of animals	Time after painting (hours)	Nuclei	Mitochondria	Microsomes	Supernatant
Mice	21	3	Dba	Dba	Dba	Dba
	27	23	Dba	Dba	Dba	Dba
Rats	8	4½	Dba	Dba	Dba	Dba
	11	17	Dba	Dba	Dba	Dba

Dba, 1 : 2 : 5 : 6 dibenzanthracene present. All fractions were isolated in 1 per cent citric acid.

TABLE XIII.—*The Removal of Polycyclic Hydrocarbons from Liver and Kidney Fractions from Rats and Mice*

Hydrocarbon	Hydrocarbon removed by: Non-polar solvents	Polar solvents	Hydrolysis
Anthracene	−	−	+
Phenanthrene	−	−	+
1 : 2 Benzanthracene	−	+	+
1 : 2 : 5 : 6 Dibenzanthracene	+	+	+
3 : 4 Benzpyrene	+	+	+

−, hydrocarbon not removed. +, hydrocarbon removed.

TABLE XIV.—*The Distribution of Polycyclic Hydrocarbons within Cell Fractions from the Liver, Kidney and Skin of Mice and Liver and Kidney of Rats*

Results apply to the first 12 hours after treatment only.

Hydrocarbon	Nuclei	Mitochondria	Microsomes	Supernatant
Anthracene	−	−	+	+
Phenanthrene	−	−	+	+
1 : 2 Benzanthracene	+	+	+	+
1 : 2 : 5 : 6 Dibenzanthracene	+	+	+	+
3 : 4 Benzpyrene	+	+	+	+

−, hydrocarbon absent. +, hydrocarbon present.

TABLE XV.—*The Distribution of Polycyclic Hydrocarbons within Cell Fractions of Rat Skin*

Results apply to the first 12 hours after treatment only.

Hydrocarbon	Nuclei	Mitochondria	Microsomes	Supernatant
Anthracene	−	+	+	+
Phenanthrene	−	+	+	+
1 : 2 Benzanthracene	+	+	+	+
1 : 2 : 5 : 6 Dibenzanthracene	+	+	+	+
3 : 4 Benzpyrene	+	+	+	+

−, hydrocarbon absent. +, hydrocarbon present.

to those obtained with the other compounds used. A typical spectrum is shown in Fig. 3.

The animals used in skin experiments received one painting with a 1 per cent solution of dibenzanthracene in acetone. Results are given in Table XII. Here again the recoveries were good and greater in quantity than with the other compounds.

Findings with dibenzanthracene resemble those with benzanthracene except that better recoveries were made and in this case "loose" hydrocarbon was present in all fractions of liver and kidney.

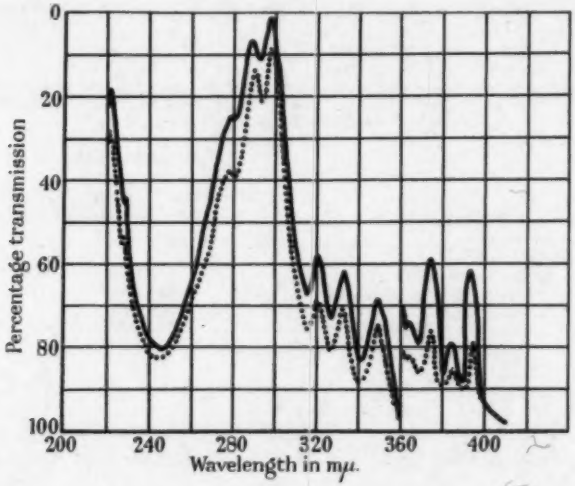


FIG. 3.—Absorption spectrum of 1 : 2 : 5 : 6 dibenzanthracene from mouse liver nuclei, 4 hours after injection of hydrocarbon.

Full line—control spectrum of dibenzanthracene in ethanol.
Dotted line—spectrum of recovered dibenzanthracene in ethanol
Above 360 mμ stronger solutions were used.

Correlation of results

The data which has been assembled above can now be added to similar data in respect of 3 : 4 benzpyrene which has already been prsented by Calcutt and Payne (1954*a*, 1954*b*) and Calcutt (1957). Considering data concerned with periods prior to disappearance of a hydrocarbon from any particular fraction a general pattern can be discerned.

In respect of the ease of removal from cell fractions the results may be summarised as in Table XIII. The findings in respect of any one hydrocarbon are the same for any cell fraction in which that hydrocarbon is found.

The distribution of hydrocarbons within cell fractions derived from mouse liver, kidney and skin and rat liver and kidney is summarised in Table XIV. The distribution within rat skin cell fractions differs slightly from that found with the other tissues and is summarised in Table XV.

DISCUSSION

The experimental findings detailed above show a remarkable distinction between the behaviour of the carcinogenic and the non-carcinogenic agents used. Immediately the question arises as to whether this is a genuine distinction or the consequence of artefact arising from the techniques used. The used of the Ultracentrifuge in biological work has been the subject of much vague criticism—without experimental support—to the effect that artefacts must be induced. Against this it must be remembered that: "strong evidence has been obtained for the absence of absorption and redistribution artefacts during cell fractionation" (Hogeboom and Schneider, 1955). Whilst evidence cannot be offered for the absence of artefacts in the present work it will, however, be shown later that the present results are consistent with the experimental findings of other biological work.

In relation to the problem of carcinogenesis the present results prove extremely interesting. Of the five hydrocarbons for which data are now available it is only the three carcinogens which are found to penetrate the nucleus. At first sight this might imply an association between carcinogenesis and action within the cell nucleus. Alternate evidence, however, does not support this. Crabtree (1946) found that anthracene or phenanthrene inhibited the induction of mouse skin tumours by 1 : 2 : 5 : 6 dibenzanthracene or 3 : 4 benzpyrene. This effect was interpreted as the consequence of competition between an active and an inactive agent for the same cellular substrate. In this event the new evidence regarding intracellular distribution would indicate either the microsomes or the supernatant as the site of such an occurrence and would accordingly implicate one of these two fractions in the carcinogenic process. Heidelberger and Moldenhauer (1956) measured the extent of binding of C^{14}-labelled 3 : 4 benzpyrene in the particulate and supernatant fractions of mouse skins and skins previously treated with agents known to reduce tumour yields (cantharidin, maleic anhydride, etc.). In the treated skins the extent of binding was reduced in both fractions but to a greater degree in the supernatant. On this basis these authors are inclined to regard the supernatant fraction as the one essentially involved in carcinogenesis.

It was shown by Creech (1939) that mouse fibroblasts subjected to 1 : 2 : 5 : 6 dibenzanthracene developed mitotic abnormalities. Under similar conditions phenanthrene was inactive. Pullinger (1940) examined the effects of a number of hydrocarbons on mouse skin and concluded that nuclear abnormalities quickly appeared after application of 3 : 4 benzpyrene or 1 : 2 : 5 : 6 dibenzanthracene but that anthracene, phenanthrene and 1 : 2 benzanthracene did not show similar effects. Ludford (1953) illustrated a range of mitotic abnormalities obtained with carcinogenic hydrocarbons. In all the above work the abnormalities showed a wide range of variation and appeared at any stage of the mitotic cycle. These points would make the production of abnormalities consistent with direct action on the nucleus rather than action mediated via the cytoplasm.

Mutations have been induced in mice by 1 : 2 : 5 : 6 dibenzanthracene (Carr, 1948) and in Drosophila with 3 : 4 benzpyrene, 1 : 2 : 5 : 6 dibenzanthracene and 1 : 2 benzanthracene but not with anthracene and phenanthrene (Demerec, 1948). As such mutations might be expected to arise as the result of nuclear

action these results are in keeping with the distributions of the hydrocarbons described above.

A rather curious feature of the results obtained is the fact that it is the hydrocarbons with the larger molecules which have shown the wider distribution within cells. If intracellular distribution is the result of a diffusion process then the smaller molecules—anthracene and phenanthrene—might have been expected to diffuse more readily than a larger molecule such as 3 : 4 benzpyrene. At the moment no information is available as to the mechanism of movement of these compounds so this problem must be left in abeyance.

SUMMARY

(*a*) The distribution of anthracene, phenanthrene, 1 : 2 benzanthracene and 1 : 2 : 5 : 6 dibenzanthracene has been determined in cell fractions from mouse or rat liver, kidney and skin.

(*b*) Anthracene and phenanthrene were only found as tightly bound material and were present in the microsomes and supernatant fractions of mouse liver, kidney and skin and rat liver and kidney. In rat skin they appeared in the mitochondria, microsomes and supernatant fractions.

(*c*) 1 : 2 Benzanthracene and 1 : 2 : 5 : 6 dibenzanthracene were found in the nuclei, mitochondria, microsomes and supernatant of liver, kidney and skin of both rats and mice.

(*d*) The experimental findings are briefly discussed in relation to the biological activity of the compounds used.

REFERENCES

BERMAN, C.—(1951) 'Primary Carcinoma of the Liver'. London (H. K. Lewis), p. 119.

CALCUTT, G.—(1957) *Brit. J. Cancer*, **11**, 605.

Idem AND PAYNE, S.—(1953) *Ibid.*, **7**, 279.—(1954*a*) *Ibid.*, **8**, 554.—(1954*b*) *Ibid.*, **8**, 710.

CARR, J. G.—(1948) *Ibid.*, **2**, 132.

CRABTREE, H. G.—(1946) *Cancer Res.*, **6**, 553.

CREECH, E. M. H.—(1939) *Amer. J. Cancer*, **35**, 191.

DEMEREC, M.—(1948) *Brit. J. Cancer*, **2**, 114.

FIALA, S., SPROUL, E. E. AND FIALA, A. E.—(1955) *Proc. Amer. Ass. Cancer Res.*, **2**, 15.

FIESER, L. F.—(1941) 'Experiments in Organic Chemistry'. 2nd Ed. New York (D. C. Heath & Co.), p. 427.

HARTWELL, J. L.—(1951) 'Survey of Compounds which have been Tested for Carcinogenic Activity'. 2nd Ed. Bethesda (National Cancer Inst.).

HEIDELBERGER, C. AND MOLDENHAUER, M. G.—(1956) *Cancer Res.*, **16**, 442.

HOGEBOOM, G. H. AND SCHNEIDER, W. C.—(1955) Chapter 21 of 'The Nucleic Acids' ed. by Chargoff, E. and Davidson, J. N. New York (Academic Press).

ILFELD, F. W.—(1936) *Amer. J. Cancer*, **26**, 743.

LUDFORD, R. J.—(1953) *J. R. micr. Soc.*, **73**, 1.

PULLINGER, B. D.—(1940) *J. Path. Bact.*, **50**, 463.

ROUS, P. AND SMITH, W. E.—(1945) *J. exp. Med.*, **81**, 597.

SHIMKIN, M. B. AND MIDER, G. B.—(1941) *J. nat. Cancer Inst.*, **1**, 707.

WIEST, W. G. AND HEIDELBERGER, C.—(1953) *Cancer Res.*, **13**, 246.